Dietary Cholesterol Metabolism in Japanese Quail Lines Selected for Plasma Cholesterol Levels

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Abstract. **Dietary cholesterol metabolism was studied, using a single dose of emulsion, *per os* (test meal), in lines of Japanese quail that were divergently selected for high (HL) and low (LL) plasma total cholesterol. The meal contained [^{3}H] cholesterol, [^{14}C] β-sitosterol, unlabeled cholesterol, triolein, and bile salt. Recovery of the non-absorbable β-sitosterol in the excreta permitted determination of the percentage of cholesterol absorbed. The amounts of [^{3}H] in the plasma, egg yolks, and the excreta neutral and acid sterols were determined. A line-x-time interaction for [^{3}H] in plasma indicated that the level of plasma cholesterol derived from the test meal declined more rapidly in the LL than in the HL. The higher [^{3}H] detected in the excreta acidic sterols of the LL 12 hr after the test meal indicated that bile acid excretion of cholesterol was greater in the LL than in the HL. There were no differences in cholesterol absorption between lines or sexes.**

Cumulative [^{3}H] radioactivity in the eggs over 18 days following the test meal was higher in the HL yolks; however, this line effect was due to the grater number of eggs produced by the HL. Thus, one of the mechanisms by which the LL maintains low plasma cholesterol levels is by an enhanced excretion of bile acid compared with the HL. The data also suggest that the more severe atherogenic effect of dietary cholesterol observed in the HL could be, in part, due to the longer residence time of cholesterol in circulation. [P.S.E.B.M. 1997, Vol 214]

The susceptibility to diet-induced hypercholesterolemia depends on several homeostatic mechanisms of cholesterol metabolism such as intestinal absorption, endogenous cholesterol synthesis, lipoprotein clearance by receptors, cholesterol removal from peripheral cells, and fecal excretion of cholesterol as neutral or acidic sterols (1–3).

Japanese quail are among the few avian species investigated that respond to exogenous cholesterol with higher plasma cholesterol levels (4–11). However, although this species has been widely used for studies of dietary-induced atherosclerosis, its parameters of cholesterol metabolism have not been determined.

Previous evidence suggests that the Japanese quail lines selected by Marks and Siegel (12) for high (HL) and low (LL) plasma cholesterol differ in cholesterol metabolism (11, 13, 14); however, the status of the regulatory mechanisms of cholesterol hemeostasis in the whole body have not been studied. We report here differences in dietary cholesterol excretion in these quail lines selected for high and low plasma total cholesterol.

Materials and Methods

Animals. Fertile eggs, obtained from the Southern Regional Genetics Laboratory (USDA, Agricultural Re-

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search Service, Athens, GA), were incubated and hatched at the Pennsylvania State University Poultry Education and Research Center (11). At 40 days of age, sex was determined and the birds were placed in individual cages. At 77 days of age, only healthy male and laying female birds (50 birds, including spares) were transferred to metabolism cages in a certified biological laboratory with the same environmental conditions. A light:dark cycle of 16:8 hr was provided throughout the experiment.

The test period was initiated when the birds were 90 days of age and continued for 18 days, at which time they were sacrificed by cervical dislocation.

Diets. An all plant source starter diet (11) and water were provided for *ad libitum* consumption from hatch to 42 days of age, after which all birds received a basal breeder diet that was enriched with 0.1% crystalline cholesterol (approx. 95% [GC], cat. no. C-8503; Sigma Chemical Co., St. Louis, MO). Birds were continued on this diet for the remainder of the experiment.

Feed Transit Time Determination. A week before administering the test meal, two birds from each line and sex were deprived of feed during the 8-hr dark period. Regular feed containing 2% chromic oxide (Fisher Scientific, Pittsburgh, PA) was provided to the birds *ad libitum* (15, 16) and the birds were monitored for the first appearance of the green chromic oxide color in the excreta. After 2 hr, the birds were placed on their regular feed.

Dosing Procedure (Single Test Meal). At 98 days of age, feed troughs were removed during the 8-hr dark period immediately preceding the administration of the single emulsion (test meal); water was continued *ad libitum*. The radioactive sterols [1α,2α(n)-^{3}H] cholesterol and β-[4-^{14}C] sitosterol were purchased from Amersham ([^{3}H] cholesterol, sp. activity, 40 mCi/m mol, cat. no. TRK-330; [^{14}C]β-sitosterol, sp. activity 55.4 mCi/m mol, cat. no. CFQ-7958; Arlington Height, IL), which were supplied as toluene and toluene-ethanol solutions, respectively, and were not further purified. Preparation of the test meal was adapted from McClelland and Shih (17). [^{3}H] cholesterol (1 μCi/bird) and [^{14}C] β-sitosterol (0.02 μCi/bird) were dried under nitrogen and redissolved with 1.2 mg unlabeled cholesterol (approx. 95%, cat. no. C-8503, Sigma) in 31.2 mg triolein (Grade 1, Sigma) per bird. The resulting oil phase was then emulsified by sonification in 0.5 ml water containing 2 mg bile salt (taurocholic acid, 99%, Sigma) per bird. Volumes were adjusted with water so that each quail received 1:1 ml *per os*, as follows: A 3-cc syringe with a stainless steel quail intubation tube was flushed with additional emulsion before use. The same syringe was used for all quail. The syringe was filled with the emulsion and 1.1 ml was delivered into the crop of each bird. The birds were not anesthetized during intubation.

Plasma Samples. Approximately 0.5 ml of blood were taken from the ulnar vein from each bird using a heparinized 1 cc syringe and a 23 G disposable needle. Only one blood sample per bird was taken during the first week after the test meal. Due to the rapid (about 75 min) feed transit time and the high metabolic rate of the quail, the first plasma sample was taken 15 min after the test meal. To obtain the disappearance curve of radioactivity in the plasma, the subsequence samples were taken based on the following geometric progression equation (18):

$$t_{(i+1)} = t_i\,(tN/t_1)^{(1/n-1)}$$

where $t_{(i+1)}$ was the next bleeding time interval, t_i was the most recent bleeding time interval, tN was the final bleeding time interval, t_1 was the time of the first bleeding, and n was the number of birds per line and sex combination. The last blood samples were taken 384 hr (16 days) after the test meal. The numbers of LL male, HL male, LL female, and HL female birds were 11, 15, 16, and 11 birds, respectively. Plasma was separated and stored at −20°C until use. Plasma samples (100 μl/sample) were added directly to 15 ml Hydroflour scintillation fluid (National Diagnostics, Atlanta, GA) and then aged 24 hr to eliminate chemiluminescence prior to counting.

Disintegrations per minute (dpm) for [^{3}H] and [^{14}C] in the samples were determined using a dual channel program in a LKB Rackbeta Scintillation Counter (LKB, Gaithersburg, MD) with quench correction. To determine radioactivity in the total plasma volume of each bird, plasma volume was calculated according to the following equation (Ref. 19, Table 5.5):

$$\text{Plasma Volume} = (0.1 \times \text{Body Weight}) \times (100 - \text{hematocrit}/100).$$

Yolk Samples. Eggs were collected the day after the test meal was given to the birds and every day for the next 17 days. Eggs were weighed, wrapped in plastic bags, and kept at 4°C until used. Yolks were carefully separated and weighed. A weighed wet yolk aliquot from each egg (approximately 200 mg) was mixed with 15 ml of scintillation fluid, aged 24 hr, and counted as described for plasma samples. Cumulative radioactivity as a fraction of dose was measured in the eggs of each bird.

Excreta Samples. Excreta samples were collected from each bird in individual trays lined with plastic bags. Excreta were collected at 2, 4, 8, and 12 hr after administering the test meal. Excreta samples from three birds chosen at random were also collected the day before the test meal; base line analyses were performed on these samples. Samples were weighed and frozen at −20°C until used. Excreta samples from each bird were pooled, thawed, and dried on a 37°C hot plate. Samples were then weighed and ground using a mortar and pestle. One to 1.5 g of each ground excreta sample was used to determine the radioactivity in the neutral and acidic sterol fractions, which were extracted according to Grundy *et al.* (16, 20) and Overturf *et al.* (21).

The samples were saponified with 1 *N* NaOH in 95% ethanol for 1 hr at 90°C. After the samples cooled below 70°C, 2 ml of water was added, and the neutral sterols were then extracted twice with hexane (5 ml each). Tubes were shaken vigorously, and partitioning of layers was carried out by low speed centrifugation (900*g*). The upper phases were pooled and measured. To determine labeled sterol content, a 1-ml aliquot was dried under nitrogen, and 15-ml of scintillation fluid was added.

The lower (aqueous) phase containing bile acids was extracted with 20 ml of chloroform:methanol solution (2:1 v/v). After 4 ml of water were added, the tube was shaken vigorously and centrifuged for 5 min at 900*g*. The lower phase was transferred to another tube and washed once with water. After discarding the upper phase, the lower phase was measured, then a 2-ml aliquot was dried under nitrogen. Four drops of 2 *N* HCl and 15 ml of scintillation fluid were added. Both neutral and acidic sterol samples were aged for 24 hr and counted as described for plasma samples. Radioactivity as a fraction of dose was also calculated. Cholesterol absorption was calculated (21):

$$\text{Percent absorption} = 1 - [(^{3}\text{H}/^{14}\text{C in Excreta Neutral Sterols})/(^{3}\text{H}/^{14}\text{C in Oral Dose})] \times 100$$

Statistical Analysis. Radioactivity data (% of dose) in the plasma were analyzed using the following models (22):

$$Y_{ijkl} = \mu + L_i + S_j + T_k + (LS)_{ij} + (LT)_{ik} + (ST)_{jk} + (LST)_{ijk} + e_{ijkl}$$

where μ is the populations mean; L_i = HL or LL populations; Sj = male or female; Tk = 0–1 hr, 1–24 hr, 1–4 days, or 4–16 days time periods; e = experimental error; and l = 3–7 quail. Plasma data were also analyzed within four time periods (0–1 hr, 1–24 hr, 1–4 days, or 4–16 days) using the following model:

$$Y_{ijkl} = \mu + L_i + S_j + B_k + (LS)_{ij} + (LB)_{ik} + (SB)_{jk} + (LSB)_{ijk} + e_{ijkl}$$

where i = HL or LL populations; j = male or female; k = first or second battery; and l = 5–10 quail.

Differences in cumulative average number of eggs between the HL and LL were tested for each day using a one-way analysis of variance (ANOVA). The covariance of egg number on cumulative radioactivity (% of dose) in the egg yolk was analyzed after removal of birds that laid fewer than four eggs. Removal was based on the fact that a third order polynomial equation best described the cumulative radioactivity appearing in the eggs. Such equations could not be constructed with less than four data points. Asymptotic values of cumulative radioactivity in the egg yolk were calculated by deriving the third order polynomial equation with the general form $y = a + bX - cX^2 + dX^3$, then taking the first differential, $dx/dy = b + 2cX + 3dX^2$, for each bird over the 18-day period, excluding those laying less than four eggs (23). General Linear Models procedures (22) were utilized to perform analyses of variance and covariance. Least square means values are reported for the various treatment combinations. Arcsine transformations of the data were also applied where appropriate.

Results

Radioactivity of [^{14}C] was not detected in plasma samples, yolk samples, or excreta acidic sterols; it was only detected in the excreta neutral sterols. Analysis of variance of [^{3}H] radioactivity (% of dose) in the plasma over a 16-day period revealed a significant time effect, but no sex or line interaction; however, the line-x-time interaction for [^{3}H] radioactivity in the plasma was significant, and these data are summarized in Table I. The decline in [^{3}H] radioactivity in the plasma in the period between the first and fourth day after the test meal was greater in the LL than in the HL quail (Table I). When the data of [^{3}H] radioactivity in the plasma were analyzed within time periods, a significant sex effect appeared only in the 4- to 14-day period following the test meal; [^{3}H] radioactivity (% of dose) that remained in the male plasma in that period was twice that of the females (0.18 ± 0.02 vs 0.09 ± 0.02, respectively).

Analysis of variance of the excreta acidic sterol data revealed no battery or sex effect, and there were no interactions among the treatment combinations; only the line effect was significant. Presence of [^{3}H] radioactivity (% of dose) in the excreta acidic sterols was higher in the LL than in the HL quail (Table II). Analysis of variance of [^{3}H] cholesterol absorption data revealed neither main effects nor interactions (Table II); the overall mean for percent cholesterol absorption was about 95%.

Figure 1 shows that the cumulative percentage of [^{3}H] radioactivity in egg yolk over 18 days following the test meal was higher in the HL than in the LL yolks. However, when covariances of egg number on [^{3}H] radioactivity in the yolk were analyzed (Table III), the line effects disappeared. The greater number of eggs produced by the HL (Fig. 2) accounted for the higher cumulative percentage of [^{3}H] radioactivity in the HL yolks.

Table I. Percentages (Least Square Means ± SEM) of Administered [^{3}H] Radioactivity Appearing in the Plasma over 16 Days following a Test Meal Containing 1 μCi [^{3}H] Cholesterol to 14-Week-Old Japanese Quail Selected for High or Low Plasma Cholesterol

Line	Time after test meal			
	1 hr	1 day	4 days	16 days
High line (HL)	0.05 ± 0.11^{a}	0.83 ± 0.10^{b}	0.61 ± 0.12^{b}	0.15 ± 0.08^{a}
Low line (LL)	0.09 ± 0.11^{a}	0.58 ± 0.09^{b}	0.19 ± 0.11^{a}	0.12 ± 0.08^{a}

^{a,b} Means having different superscripts are significantly different ($P < 0.05$).

Table II. Percentages (Least Square Means ± SEM) of [^{3}H] Cholesterol Absorption and Percentages of Administered [^{3}H] Radioactivity Appearing in the Fecal Acidic Sterols 12 hr following a Test Meal Containing 1 μCi [^{3}H] Cholesterol to 14-week-old Japanese Quail Selected for High or Low Plasma Cholesterol

	n	[^{3}H] cholesterol absorption (%)	[^{3}H] in fecal acidic sterols (% of dose)
Line			
High line (HL)	26	95.53 ± 0.61	1.00 ± 0.33^a
Low line (LL)	27	94.24 ± 0.59	1.87 ± 0.32^b
Sex			
Male	26	94.60 ± 0.62	1.79 ± 0.33
Female	27	95.17 ± 0.58	1.07 ± 0.31

^{a,b} Means within column subgroups having different superscripts are significantly different ($P < 0.05$).

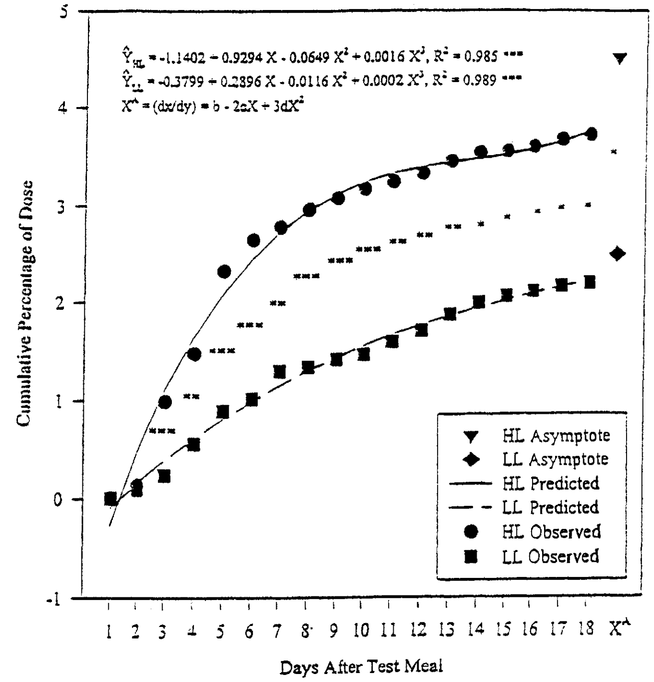

Figure 1. Cumulative percentage of administered [^{3}H] radioactivity appearing in egg yolk over 18 days following a test meal containing 1 μCi [^{3}H] cholesterol to 14-week-old Japanese quail selected for high (HL) or low (LL) plasma cholesterol. ***,**,*$P < 0.001, 0.01, 0.05$, respectively.

Discussion

The results of the present study indicate that one of the mechanisms by which the LL maintains low plasma cholesterol levels is by greater bile acid excretion compared to the HL. Cholesterol absorption, synthesis, and turnover have been studied in selected lines of hypo- and hyper-responding Show Racer pigeons by feeding a diet containing [^{3}H] cholesterol and injecting a [^{14}C] cholesterol labeled serum (24). There was greater removal from the cholesterol pool by the hypo-responders, signifying genetic control of plasma cholesterol at the level of excretion. Overturf *et al.* (21) studied dietary cholesterol absorption, and sterol and bile acid excretion in hypercholesterolemia-resistant New Zealand white rabbits using a single test meal containing [^{3}H] cholesterol and [^{14}C] β-sitosterol, and found that the fecal bile acid excretion of the hypercholesterolemia-resistant rabbits was more than twice as great as that of the "normoresponsive" rabbits. Green *et al.* (18) investigated cholesterol turnover and tissue distribution in the guinea pig and found that the maintenance of cholesterol homeostasis in the nonhypercholesterolemic cholesterol-fed guinea pig depends on liver accumulation of esterified cholesterol as well as an increased output of cholesterol. Although increasing bile acid excretion was shown to be one mechanism by which low plasma cholesterol levels are maintained in the LL quail, other mechanisms such as cholesterol storage in the liver and endogenous cholesterol synthesis remains to be investigated in the selected quail lines.

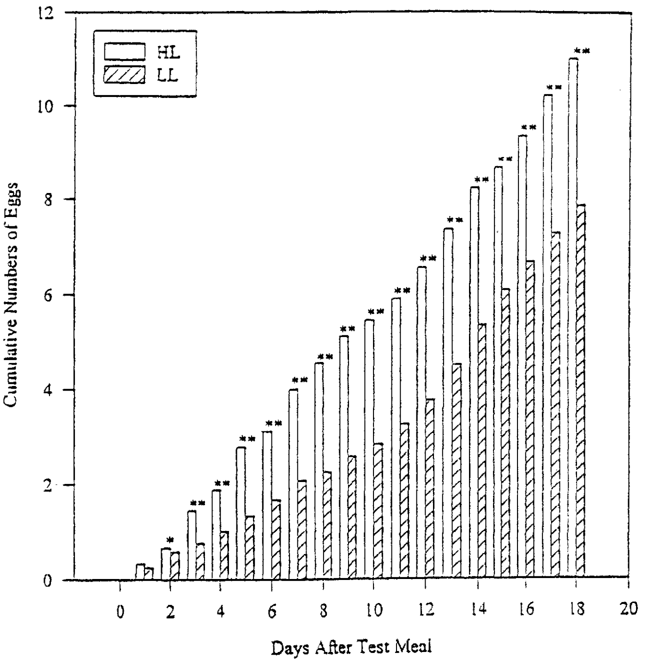

Figure 2. Cumulative average number of eggs produced by females of Japanese quail selected for high (HL) or low (LL) plasma cholesterol following a test meal containing [^{3}H] cholesterol. **,*$P < 0.01$, 0.05, respectively.

Information about cholesterol metabolism in Japanese quail is limited. Accumulation of cholesterol in serum, liver, and aorta was evaluated in 5-week-old male Japanese quail fed an "inducer" diet with 1% cholesterol (25). The cholesterol concentrations of the serum and liver of quail fed the inducer diet were 6.7 and 14.4 times greater, respectively, than those of the control group. Moderate to severe lipid accumulation was observed in the aortae of the inducer-diet-fed quail at 10 weeks of age. McClelland and Shih (17) used a dual isotope technique to measure the rates of cholesterol absorption and fecal neutral acidic sterol excretion in Japanese quail fed purified diets containing dif-

Table III. Analyses of Covariance for Effect of Egg Number on Cumulative Percentage of Administered [^{3}H] Radioactivity Appearing in Egg Yolk over 18 Days following a Test Meal Containing 1 μCi [^{3}H] Cholesterol to 14-week-Old Japanese Quail Selected for High (HL) or Low (LL) Plasma

Source of variation	*DF*	Days after test meal																		
		1 ($\times 10^6$)	2 ($\times 10^6$)	3 ($\times 10^3$)	4 ($\times 10^3$)	5 ($\times 10^3$)	6 ($\times 10^3$)	7 ($\times 10^3$)	8 ($\times 10^3$)	9 ($\times 10^3$)	10 ($\times 10^3$)	11 ($\times 10^3$)	12 ($\times 10^3$)	13 ($\times 10^3$)	14 ($\times 10^3$)	15 ($\times 10^3$)	16 ($\times 10^3$)	17 ($\times 10^3$)	18 ($\times 10^3$)	$x^{\infty a}$ ($\times 10^3$)
Total		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
I^b Line	1	0.13	0.12	1.43**	1.44**	2.57**	3.01**	1.86**	209**	1.80**	1.84**	1.37**	1.11**	1.01**	0.92*	0.82*	0.78*	0.79*	0.80*	0.84*
Egg no.	1	44.75**	10.38**	3.37**	5.87**	6.30**	8.16**	7.51**	8.45**	7.99**	8.31**	7.22**	6.67**	3.74**	2.77**	2.31**	2.31**	1.85**	1.53**	1.62**
III^c Line	1	0.06	0.02	0.20	0.16	0.29	0.17	0.00	0.02	0.07	0.04	0.04	0.06	0.00	0.00	0.04	0.04	0.06	0.09	0.11
Egg no.	1	44.75**	10.38**	3.37**	5.87**	6.30**	8.16**	7.51**	8.45**	7.99**	8.31**	7.22**	6.67**	3.74**	2.77**	2.31**	2.31**	1.85**	1.53**	1.62*
Error	18	0.84	0.21	0.06	0.10	0.17	0.13	0.13	0.11	0.11	0.11	0.12	0.12	0.12	0.13	0.15	0.15	0.16	0.17	0.19

Note. Values in table body, with the exception of *DF* values, are mean squares (arcsine $\sqrt{\%}$).

[a] Asymptotic value.

[b] Type I means squares indicate non-covariate effects of egg number on cumulative percentages.

[c] Type III means squares indicate covariate effects of egg number on cumulative percentages.

**,* $P < 0.01$, 0.05, respectively.

ferent levels of soy protein either with or without 0.5% cholesterol. The results of their study demonstrated that the presence of high (40%) dietary levels of soy protein had both a hypocholesterolemic action and a preventive effect on cholesterol-induced atherosclerosis; one of the possible mechanisms was through increased excretion of cholesterol. The kinetic study by McClelland and Shih (17) on quail showed that the isotope ratio ([^{14}C]/[^{3}H]) became constant by the fourth day after dosage. The time periods in the present study were selected on that basis. The average transit time through the quail gastrointestinal tract in this study was found to be about 75 min. That finding was also taken into consideration when time intervals for blood sampling were selected. The fact that [^{3}H] radioactivity was higher in the male than female plasma in the 4- to 16-day period following the test meal could be explained by the transfer of the exogenous cholesterol to the egg yolk in the females. However, the reason the difference was not observed earlier can not be fully explained at this point. Japanese quail hens require about 6 days (26) for the rapid development of ovarian follicles and an additional day for egg formation. When and to what extent dietary cholesterol, including the radioactive [^{3}H] tracer, was exchangeable with endogenous cholesterol in general, and ovarian cholesterol in particular, is not clear.

In contrast to the study by McClelland and Shih (17), who administered [^{14}C] cholesterol orally and [^{3}H] cholesterol intravenously to determine cholesterol absorption by the plasma isotope ratio methods, in the present study cholesterol absorption was quantified by using an oral dose containing [^{3}H] cholesterol and [^{14}C] β-sitosterol, which was used as an internal standard to correct for cholesterol losses (2). Dam *et al.* (27) studied the effect of β-sitosterol on egg cholesterol deposition in Japanese quail and found no increase in yolk cholesterol values when 2% β-sitosterol was added to the diet. Furthermore, these authors found no sitosterol in the yolk. In the present study, the radioactivity of [^{14}C], whose source was [^{14}C] β-sitosterol, was not detected in plasma samples, yolk samples, or excreta acidic sterols.

The data from the present study indicate that the more severe atherogenic effect of dietary cholesterol observed in the HL (14) is, in part, due to the longer residence time (Table I) of cholesterol in circulation. Groot *et al.* (28) reported that patients with coronary artery disease had a delayed clearance of postprandial lipoproteins from their serum.

An interesting finding of the present study is that the HL females produced more eggs than the LL females between 14 and 16 weeks of age, which led to the presence of higher cumulative [^{3}H] radioactivity in the HL yolks. In previous studies (29), it appeared that dietary cholesterol might be necessary for optimum egg production in the HL quail.

Kuan and Dupont (30) studied dietary cholesterol effects on cholesterol metabolism in CBA/*J* and C57BR/*cdJ* strains of mice, which are resistant and susceptible to dietary-induced hypercholesterolemia, respectively. They reported that the two strains maintained cholesterol homeostasis by different mechanisms. The CBA/*J* mouse adjusted hepatic HMG CoA reductase activity, and the C57BR/*cdJ* mouse changed fecal excretion of cholesterol. Whether the selected Japanese quail lines respond to dietary cholesterol with different homeostatic mechanisms remains to be studied.

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