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Physiological Response of Layers to Alternative Feeding Regimen of Calcium Source and Phosphorus Level

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Abstract: An experiment was conducted to determine the effects of dietary calcium source, phosphorus level and the time of feeding on the physiological responses of laying hens pertaining to egg shell formation. Seventy-two Hyline W-36 hens were divided into three treatment groups. Treatment 1 was fed a commercial layer ration as morning and evening feed with limestone as the calcium source. Treatment 2 was fed the commercial ration in the morning, while the evening feed contained a normal calcium level with limestone as the calcium source, but a lower phosphorus level than Treatment 1. Treatment 3 feed contained normal calcium and phosphorus in the morning while Treatment 3 evening feed contained normal calcium, but low phosphorus. The calcium source for Treatment 3 was ½ limestone and ½ oyster shell. Blood was drawn for each post-oviposition hour from all three-treatment groups to collect 4 samples per hour. Oyster shell or the reduced phosphorus level in the layers' diet did not raise the plasma calcium level in Treatment 2 or 3 compared to Treatment 1 that had a significantly higher plasma calcium level. Reduced dietary phosphorus decreased plasma inorganic phosphorus in Treatment 3. Limestone alone with reduced phosphorus (Treatment 2) did not decrease plasma phosphorus. Reduced dietary phosphorus and/or calcium source appears to have affected blood levels of estradiol and progesterone. Eggshell quality can be improved by partial (½) replacement of the limestone with Oyster shell. Reduced phosphorus did not improve eggshell quality, however, it did help indirectly by reducing plasma inorganic phosphorus, the excess of which is otherwise detrimental to eggshell quality.

Key words: Blood calcium, phosphorus, estradiol, progesterone, alternative feeding, calcium source

Introduction

Poor eggshell quality has been a major economic concern to commercial egg producers with an estimated annual loss of around $478 million (Roland, 1988). There are a number of factors involved in eggshell formation and its subsequent quality (Butcher, 1996). The macro factors include, but are not limited to, the source and level of calcium in the diet, phosphorus level in the diet and temporal intake of these minerals. The source and particle size of calcium used in laying hen diets are two factors that have received considerable attention (Roland, 1986; Rao and Roland, 1989). Oyster shell and limestone are the two principal sources of calcium used in laying hen rations. Different aspects of shell quality were reported to be improved by partial replacement of limestone with oyster shell in the diet (Moran *et al*., 1970; Scott *et al*., 1971; Brister *et al*., 1981; Makled and Charles, 1987).

Phosphorus is an important mineral for eggshell formation. Eggshells contain very little phosphorus (calcium: phosphorus in eggshell is approximately 100:1), but this element interacts with calcium for bone formation. Calcium is stored in the skeleton almost entirely as calcium phosphate; therefore, synthesis of medullary bone requires dietary phosphorus. This phosphorus is, however, involved in an essentially "futile" process, because if the calcium is used for shell formation, the phosphorus must be excreted. Nutritional interest in phosphorus has been stimulated by several observations that dietary excess of this element has a detrimental effect on shell quality (Arscott *et al*., 1962; Taylor, 1965; Harms, 1982a, 1982b). It is not clear whether this phosphorus excess, by accumulating in the blood, interferes with mobilization of skeletal reserves of calcium phosphate during shell formation in the dark or whether there is a direct antagonistic effect of blood phosphorus on the shell forming process. Whatever the mechanism, there is no doubt that dietary treatments which lead to an increase in plasma phosphate, cause a decline in egg specific gravity and thus the shell quality. Miles and Harms (1982) showed a clear negative linear correlation between specific gravity and plasma phosphate over a range of treatments. The National Research Council (NRC, 1971, 1977) recognized the earlier reports and lowered its recommended total phosphorus level for laying hens from 0.60 to 0.50%. Other reports indicate that the minimum total phosphorus requirement of the laying hen is below 0.50% (Hamilton, 1980; Miles *et al*., 1983). Mongin and Sauveur (1974) observed that higher

calcium consumption takes place early in the morning and late in the afternoon, primarily on days concurrent with ovulation and oviposition. Hens became deficient in calcium during the early morning hours and the time of calcium intake was important in the ability of the hen to calcify eggshells (Scott *et al.,* 1971; Lennards *et al.*, 1981). The importance of time of calcium intake was believed to be due to the inability of the hen to maintain adequate calcium reserves in the small intestine during the period of shell formation (Roland and Harms, 1973). It is further reported that the period of greatest calcium deficiency in the digestive tract of the laying hen was between 2400 and 0400 hours. It must be kept in mind that during this period, in the early morning hours, eggshell calcification is usually occurring at a very rapid rate. During this phase of the hens' daily egg laying cycle, calcium and phosphorus are being mobilized from bone and the phosphorus level in the blood begins to increase. Peak plasma and organic phosphorus concentration 15 hours after ovulation might be attributable to medullary bone resorption during shell formation (Van de Velde *et al.*, 1986). It is speculated that this rise in blood phosphorus level interferes, through some mechanisms with calcification of eggshell, on one hand and taxes the hen's body through excess excretion on the other. The aim of the present study was to observe and analyze the effects of calcium source, phosphorus level and changing the phosphorus level in the evening feed of the layers at the same time to understand their interaction and influence on plasma concentrations of calcium, inorganic phosphorus, estradiol and progesterone.

Materials and Methods

Seventy-two Hyline W-36 hens were divided into three treatment groups of 24 hens. Each hen was housed in a separate individual cage. Treatment 1 was fed a commercial layer ration as morning and evening feed with limestone as the calcium source and served as the control. Treatment 2 was fed the commercial ration (control) in the morning, while the evening feed contained a normal calcium level with limestone as the calcium source, but a lower phosphorus level than control feed. Treatment 3 feed contained normal calcium and phosphorus in the morning while Treatment 3 evening feed contained normal calcium, but low phosphorus (same level as Treatment 2 evening feed). The calcium source for Treatment 3 was ½ limestone and ½ oyster shell. The morning feed was fed at 8:00 AM, while the evening feed was fed at 4:00 PM. The feeders were thoroughly cleaned from the residual feed before the next feed was offered. The experimental feed formulation, tabulated ingredient composition and chemical analysis of the feeds are given in Tables 1, 2, and 3, respectively. All treatments were initiated at about 28 weeks of age when the birds had achieved their peak egg production. The birds in all three-treatment groups were fed a commercial layer ration from 18-28 weeks. Feed and water were provided for *ad libitum* consumption throughout the experiment. The incandescent light was provided 16 hours per day from 8:00 AM to midnight. The bulbs were turned off by time clock at midnight until 8:00 AM.

Blood collection: On blood collection days each bird was observed from 7:00 AM until 12:00 noon for oviposition. Once a bird laid an egg, the time was recorded on a card hanging in front of her cage and also on the egg. The particular bird then was bled for different post oviposition times by lateral cardiac puncture (approved by the Laboratory Animal Care Office, Michigan State University). On each occasion, 3 ml of blood was drawn into a heparinized syringe to avoid any clotting (green light was always used in the dark phase during blood collection to minimize any disturbances to the birds). Lithium heparin was utilized to avoid assay problems of the plasma. Blood samples were collected from individual birds only once per 7 day period to avoid hemodilution. This process was repeated until 4 samples at each hour post oviposition from 0 to 24 hours were collected from each Treatment. There were 300 total blood samples (4 sample/hr; 100 sample/Treatment).

The blood samples were dispensed from the syringe into heparinized test tubes and were centrifuged at 2000 g for 10 minutes. The plasma was removed and stored in glass vials in a freezer at -10 ^{o}C for subsequent analysis.

Plasma calcium: The plasma calcium at each hour post-oviposition was determined using an atomic absorption spectrophotometer with an air-acetylene flame (Perkin-Elmer, 1982). Lanthanum chloride was used as sample diluents to control the chemical interferences (strong phosphate interference) in the calcium determination.

Plasma inorganic phosphorus: The plasma inorganic phosphorus at each hour post oviposition was quantitatively determined using a kit for inorganic phosphorus determination (Procedure No. 670, Sigma Chemical Co. P.O Box 14508, St Louis, MO 63178.)

Procedure: One half ml of chicken blood plasma was pipetted into a borosilicate glass tube. Two and a half ml of water and 2.0 ml of trichloroacetic acid (20% w/v) were added into the tube. The contents were thoroughly mixed and then centrifuged for 10 minutes at 2000 g to obtain a "clear solution." The "test" was prepared, from 2 ml of this solution, by adding 3 ml of water and 1 ml of acid molybdate solution. A "blank" was prepared using 2 ml of trichloroacetic acid, instead of the "clear solution." The remaining reagents (water and acid molybdate) were

added in the same volumes. One-quarter ml of Fiske and Subba Row solution was added to both the 'test' and "blank" mixed and left undisturbed for 10 minutes for color development. The contents of the "test" and "blank" were then transferred into cuvets and the absorptance (A) of the 'test' was recorded at 660 nm using the "blank" as the zero reference. A Perkin-Elmer Junior III spectrophotometer was utilized for this. The inorganic phosphorus concentration of the "test" was determined from a standard curve prepared at the same time.

Plasma estradiol-17β: Plasma estradiol-17β was determined using an ICN Biomedicals, (ICN Biochemicals, Inc. 3300 Hyland Ave, Costa Mesa, CA 92626) Inc. kit employing ^{125}I radioimmunoassay techniques.

Assay: This was a 90-minute incubation, with 50-ul-sample size assay. All the reagents were brought to room temperature prior to their use and were directly pipetted from their vials. The assay was done in duplicate.

A standard curve was prepared utilizing 50 ul of estradiol standards (0, 10, 30, 100, 300, 1000 and 3000 pg/ml) supplied with the assay kit. Instructions of the kit were followed precisely and the final product was counted in a gamma counter (1290 Gamma Trac, TM Analytic, Elk Grove Village, IL.) calibrated for ^{125}I. All tubes were counted for one minute. The results were calculated as follows.

Average counts were calculated for all duplicates. Average non-specific binding counts were subtracted from the averages calculated above to give corrected counts. These corrected counts were divided by the corrected B_0 counts to obtained the percent bound by the following formula:

$$\text{Percent bound} = \frac{\text{CPM (sample) - CPM (NSB)}}{\text{CPM (O pg/ml) - (CPM (NSB)}} \times 100$$

CPM = Average counts per minute of duplicate tubes.

Sample = Particular plasma at each hour post oviposition.

NSB = Non-specific binding tube (no standard or anti-E2 or antibody added to tube) for background counts.

B_0 = Total binding tube or 0 pg/ml tube of standard curve.

= CPM (0 pg/ml) - CPM (NSB)

Example:

10mg Calculation = $\frac{17350-1000}{18298-1000} \times 10$

% B/Bo = 95%

A plot of the percent bound (Y-axis) versus concentration of the estradiol standards (X-axis) starting with 10-pg/ml point was constructed. Using this standard curve, estradiol concentrations at each hour post oviposition (4 sample/hr) were determined. A new standard curve was obtained at each time the assay was performed.

Plasma progesterone: Plasma progesterone of the chickens in the trial was determined using the ICN Biomedicals, Inc. kit employing ^{125}I radioimmunoassay techniques. Essentially the same procedure and equipment were used for that as for the estradiol assay, except the sample size and incubation time, which were 100 ul and 60 minutes, respectively. Since these kits were basically developed for human estradiol and progesterone quantification, both the assays were validated for chicken plasma by parallelism and recovery of estradiol and progesterone from chicken plasma.

Statistics: Plasma calcium, phosphorus, estradiol and progesterone were analyzed by factorial analysis of variance using repeat measurements with time and Treatment as the main factors. The different means were analyzed by the Tukey-Kramer Honestly Significant Difference (Tukey, 1953).

Results and Discussion

Plasma calcium: The concentrations of plasma calcium of the three Treatments, as determined by atomic absorption spectrophotometry are presented in Fig. 1. The data thus obtained (4 observations/hr) was averaged from each hour in each treatment and analyzed as repeat measurements by factorial analysis of variance with time and treatments as the main factors. There was a significant difference ($P<0.001$) in time, treatment ($P<0.05$) and time by treatment ($P<0.001$) interaction. Treatment 1 had the highest plasma calcium level at 0 hr among the three treatments, reached its peak level (37.07 mg/dl) at 10 hr and then declined towards its lowest level at 22 hr (17.61 mg/dl) post oviposition. Treatment 2 had the lowest plasma calcium level among the 3 treatments at 0 hr (15.58 mg/dl), reached maximum at the 9 hr (31.49 mg/dl) and then dropped to its lowest at 17 hr (15.71 mg /dl) post oviposition. Treatment 3 had its lowest plasma concentration at the 8 hr (14.47 mg/dl), started rising and reached to its peak concentration at the 21 hr (30.22 mg/dl) post oviposition with fluctuations in between hours. At 16 hr post oviposition we observed an uncharacteristic plasma calcium level of 51.85 mg/dl, which we believe an outlier and have not included in the analysis. These calcium concentrations are not in agreement with those of Taylor (1970) who reported that the average serum calcium level among producing hens varies from approximately 20 to 30 mg %. This difference in results could be due to the changes in experimental diets in the present study and the average NRC requirements two decades ago. The bird's genetics and

Table 1: Experimental Rations

Ingredients	Ration 1	Ration 2	Ration 3
Corn	60.85	61.15	61.15
Soybean meal (44%)	25.00	25.00	25.00
Dried alfalfa meal	2.50	2.50	2.50
Fat	0.50	0.50	0.50
Vitamin mineral premix	0.35	0.35	0.35
DL-methionine	0.10	0.10	0.10
Salt	0.40	0.40	0.40
Limestone	8.60	9.00	4.50
Oyster shell	0.00	0.00	4.50
Dicalcium phosphate	1.70	1.00	1.00
TOTAL	100.00	100.00	100.00
Calculated Analysis			
Met. Energy Kcal/Kg	2666	2676	2676
Crude protein %	16.77	16.80	16.80
Crude fiber %	3.76	3.77	3.77
Calcium %	3.75	3.75	3.75
Total phosphorus %	0.66	0.53	0.53
Available phosphorus %	0.45	0.32	0.33

Ration 1= control, normal calcium and phosphorus, Ration 2 = normal calcium (all limestone), low phosphorus, Ration 3 = normal calcium (½ limestone & ½ Oyster shell), low phosphorus, Ca = calcium, P = phosphorus, LS = limestone, OS = Oyster shell

Table 2: Tabulated Chemical Composition of the Experimental Rations

Ingredients	Percent	ME Kcal/Kg	CP %	CF %	Ca %	TP %	Av. P %
Corn	60.85	2030	5.3320	1.3332	0.01212	0.16968	0.0606
SBM 44%	25.00	558	11.0000	1.8250	0.07250	0.16250	0.0675
Alfalfa	2.50	34	0.4375	0.6025	0.03600	0.00550	0.0055
Fat	0.50	44					
Premix	0.35						
DL-methionine	0.10						
Salt	0.40						
Limestone 38%	8.60				3.2680		
Dical.	1.70				0.3621	0.3179	0.3179
TOTAL	100.00	2666	16.7703	3.7597	3.7507	0.6556	0.4515
Ration 2 (low available P (0.30%) with LS as Ca)							
Dical.	1.00				0.2130	0.1870	0.1870
Limestone	9.00				3.4200		
Corn	61.15	2060	5.3592	1.3398	0.01218	0.1705	0.0690
TOTAL	100.00	2676	16.7967	3.7663	3.75366	0.5255	0.3209
Ration 3 (low available P with ½ LS & ½ OS as Ca)*							
Limestone	4.50				1.7100		
Oyster shell	4.50				1.7100		0.0045
TOTAL					3.4200		0.3254

* All the analysis for ration 3 is otherwise the same as ration 2, except available phosphorus and calcium source. ME = metabolizable energy, CP = crude protein, CF = crude fiber, Ca - calcium, TP = total phosphorus, Av. P = available phosphorus, SBM = soybean meal, Premix = vitamin and mineral premix, Dical. dicalcium phosphate

production potentials might be another reason. The present results are somewhat similar to those of Lennards *et al.* (1981) who found no correlation between serum calcium and shell weight or egg weight, but serum calcium was positively correlated to egg specific gravity in one of their three experiments. They concluded that the normal variation in serum calcium is not related to the hen's ability to produce eggshell. In the present study, Treatment 3, which showed the largest variation in plasma calcium, also had the highest egg specific gravity as compared to Treatment 1, and 2 without any significant difference in egg weight or eggshell

Table 3: Proximate Analysis of the Experimental Rations (Two Batches)

Nutrients	Ration 1		Ration 2		Ration 3	
%	batch 1	batch 2	batch 1	batch 2	batch 1	batch 2
Moisture	7.50	10.10	7.60	7.90	8.00	9.90
Crude Protein	17.10	16.70	16.80	17.20	16.00	16.80
Crude Fat	2.80	2.70	2.80	2.60	2.80	2.30
Acid-detergent Fiber	4.70	4.60	5.40	4.70	5.20	5.00
Calcium	4.38	4.34	4.34	4.20	4.18	4.30
Phosphorus	0.68	0.71	0.51	0.50	0.51	0.65

Ration 1 = control, normal calcium and phosphorus, Ration 2 = normal calcium (all limestone), low phosphorus, Ration 3 = normal calcium (½ limestone & ½ Oyster shell), low phosphorus

thickness.

When examined across each hour, Treatment 3 had the lowest plasma calcium, except at the 16th hr as compared to the control diet (Treatment 1) that we believe an outlier. On the average, Treatment 3 had 22.62-mg/dl plasma calcium vs. 25.63 mg/dl of Treatment 1, while Treatment 2 had 23.68-mg/dl plasma calcium across the 24 hours post oviposition range. Treatment 1 was significantly different ($P<0.05$) from Treatments 2 and 3 for plasma calcium, while Treatments 2 and 3 were not different from each other for plasma calcium. As such, these results are not in agreement with the published reports (Van de Velde *et al.*, 1986; Cheng and Coon, 1990; Guinotte and Nys, 1991) where no difference was found in plasma calcium of laying hens when Oyster shell was added or replaced limestone.

The overall difference in plasma calcium across the oviposition-ovulation cycle does not tell the whole story. The comparative changes in the concentration of plasma calcium at the time of eggshell formation bears more importance whether a particular treatment is helpful in this important physiological function. Although Treatments 2 and 3 had lower average plasma calcium concentrations during the 24 hours post oviposition time, the variation in the concentration of Treatment 3 tells a different story. It is hard to establish any definitive correlation between this and eggshell quality and hen performance traits, yet apparently this looks responsible for improved specific gravity and egg production of Treatment 3 hens.

Plasma inorganic phosphorus: The average concentrations of plasma inorganic phosphorus at each hour post oviposition are presented in Fig. 2. The data was analyzed by factorial analysis of variance using repeat measurements with treatment and time as the main factors. The overall Treatment means were 5.53, 5.20, and 4.19 mg/dl, respectively, for Treatments 1, 2, and 3. There was a significant difference ($P<0.01$) among treatments, time and the treatment by time interaction. Treatment 3 had a lower ($P<0.05$) concentrations of plasma inorganic phosphorus compared to Treatments 1 and 2. Treatments 1 and 2, however, were not different from each other. Treatment 3 had the lowest plasma inorganic phosphorus concentration at 0 hr post oviposition among the three treatments. At 5 hr post oviposition it reached its minimum value of 2.99 mg/dl and started rising. It reached its peak value of 5.84 mg/dl at 15 hr and then once again started dropping until it reached 4.10 mg/dl at 24 hr post oviposition. The birds on control diet (Treatment 1) behaved very differently compared to Treatment 3 birds. They (Treatment 1) had their peak plasma inorganic phosphorus concentration of 7.43 mg/dl at the 5 hr post oviposition when Treatment 3 birds had their minimum value of 2.99 mg/dl. This value (7.43 mg/dl) started declining and at 22 hr post oviposition, reached the minimum value of the cycle (3.80 mg/dl). Treatment 2 had a zigzag fluctuation throughout a 24-hr post oviposition cycle with a maximum of 8.24 mg/dl at 9 hr and a minimum value of 3.51 mg/dl at 19-hr post oviposition. A definitive pattern within each Treatment and a relationship among Treatments is not apparent.

One explanation for this variation in plasma phosphorus concentration at each hour could be that all the 24 birds in each Treatment were bled periodically for each specific hour rather than one bird for each different hour post oviposition.

All 3 Treatments had one initial peak at around 2-3 hr post oviposition before reaching their final maximum values. After 15 hr all Treatments started declining towards their minimum values near 22-25 hr post oviposition.

These results are in partial agreement with those of Van de Velde *et al.* (1986) who obtained large and significant individual variation in total plasma phosphorus concentration. In their experiment inorganic plasma phosphorus concentration reached a peak of 6.9 mg/dl at 15 hr after ovulation and fell to 6.0 mg/dl at 22 hr after ovulation. In the present study, Treatment 3 reached the

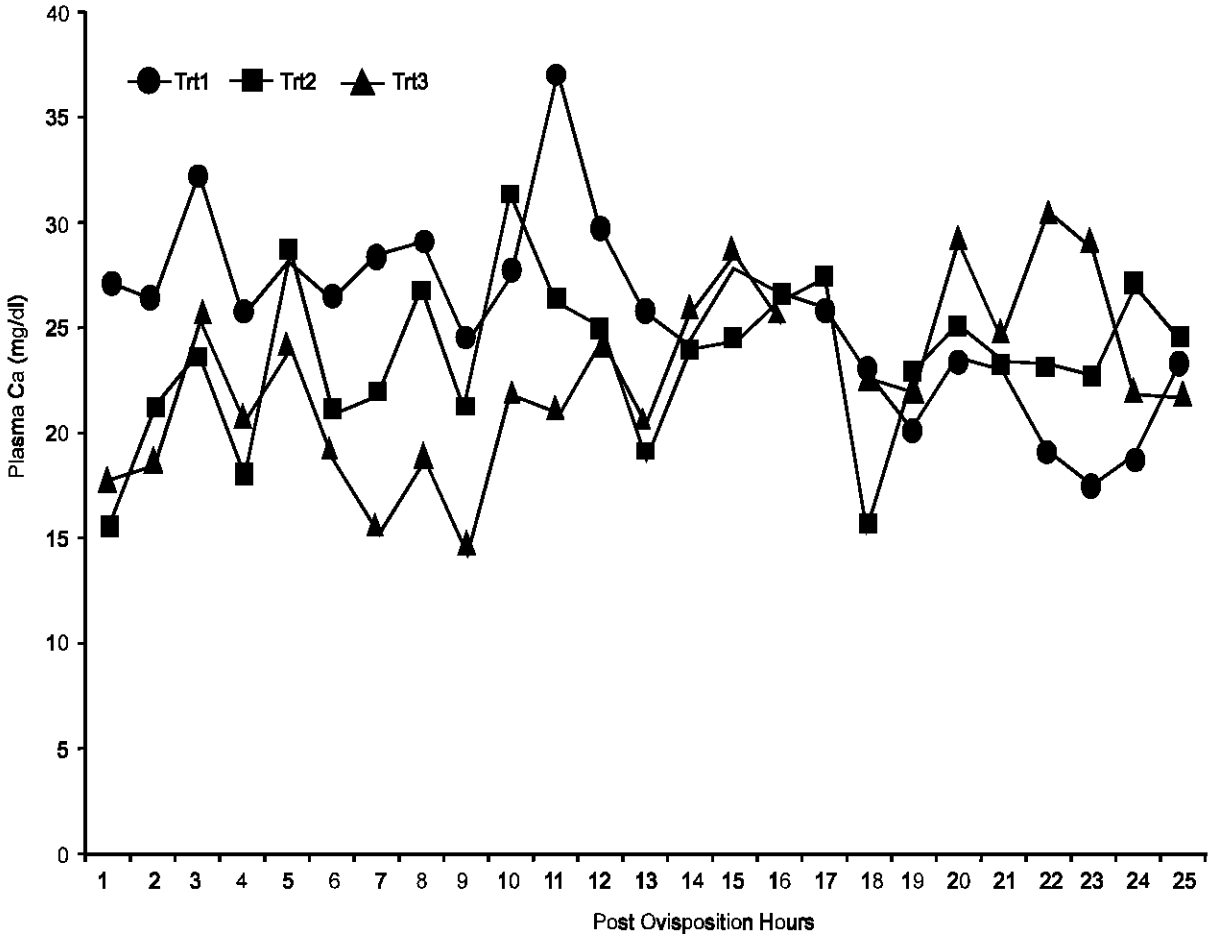

Fig. 1: Mean plasma calcium concentration (mg/dl) at hourly intervals post oviposition in laying hens fed control diet (Treatment 1), limestone with reduced phosphorus in the evening feed (Treatments 2) or ½ limestone and ½ oysteshell with reduced phosphorus in the evening feed (Treatments 3)

peak concentration of plasma inorganic phosphorus at 15 hr post oviposition that was 14-14.5 hr after ovulation and reached its lower level at 24 hr post oviposition that was 23-23.5 hr after ovulation. The other two Treatments did not follow the peak time, although the minimum concentration time was about the same. These results are also in somewhat agreement with those of Frost and Roland (1991) who reported significantly decreased plasma phosphorus with decreasing dietary phosphorus concentration. The peak plasma phosphorus level in their experiment was observed at 16-hr post oviposition. Treatments 2 and 3 of the present study contained reduced dietary phosphorus levels compared to the control diet (Treatment 1), but had a different calcium source. This could be one reason for the discrepancy in the results between the present study and that of Frost and Roland (1991). Although Treatment 3 had lower plasma phosphorus level as compared to the control diet and peaked at 15 hr post oviposition; Treatment 2 was not significantly different from the control and peaked at 9 hr post oviposition. The reduced plasma phosphorus concentration of Treatment 3 might be due to synergistic effects of Oyster shell and reduced dietary phosphorus as Oyster shell tended to be more available during shell formation, thereby reducing resorption from bone, which in turn avoided extra phosphorus flow into blood. These results are also in partial agreement with Keshavarz (1986) who used three available phosphorus levels of 0.24, 0.44 and 0.64% along with 3 calcium levels of 3.5, 4.5, and 5.5% in the diets of 56 weeks old commercial laying hens for 16 weeks. Plasma phosphorus and eggshell quality were not significantly affected by dietary levels of phosphorus except that the lowest dietary phosphorus had lower plasma phosphorus. These results are in agreement with other reports (Miller *et al.*, 1977; Mongin and Sauveur, 1979) that confirmed the cyclic nature of the serum phosphorus in relation to the egg laying cycle. These reports further confirmed the sharp reduction in the serum inorganic phosphorus concentrations 2 to 3

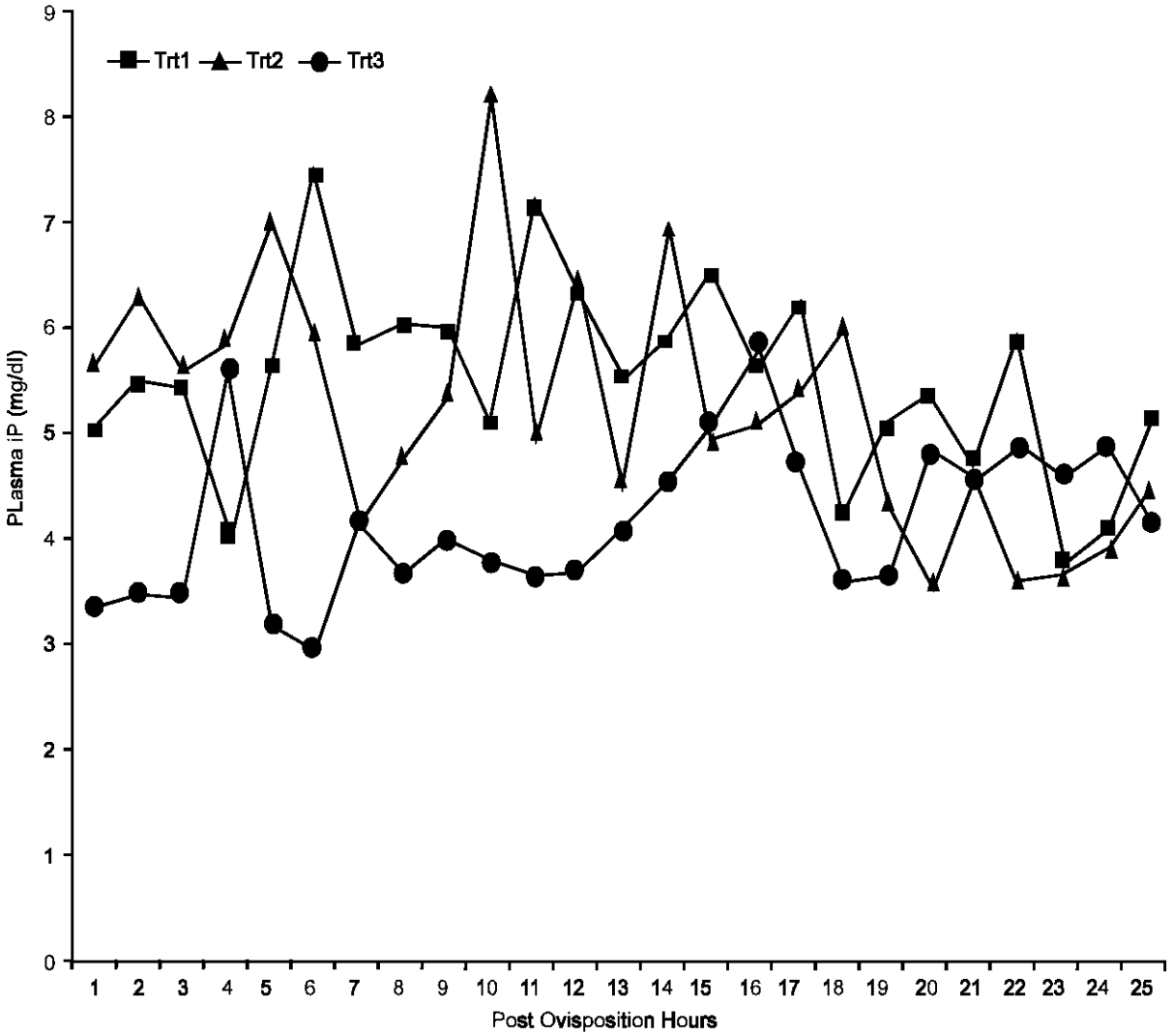

Fig. 2: Mean plasma inorganic phosphorus concentration (mg/dl) at hourly intervals post oviposition in laying hens fed control diet (Treatment 1) limestone with reduced phosphorus in the evening feed (Treatments 2) or ½ limestone and ½ oysteshell with reduced phosphorus in the evening feed (Treatment 3)

hr prior to oviposition.

Plasma estradiol: The average concentration of plasma estradiol at each hour post oviposition is presented in the Fig. 3 (Each hour in Fig. 3 in all three treatments represent an average of 4 samples per hour with duplicate analyses per sample). The data was statistically analyzed by factorial analysis of variance using repeat measurements with treatment and time as the main factors. The overall treatment means were 399.3, 519.7 and 410.9 pg/ml respectively, for Treatments 1, 2 and 3. There was a significant difference ($P<0.001$) among treatments, time and the treatment by time interaction. Treatment 2 was higher ($P<0.05$) than Treatments 1 and 3. Treatments 1 and 3 were not different from each other. There was an obvious trend among the treatments; all three had peak values at 20-22 hr post oviposition and then sharply dropped near the time of the next oviposition. Treatment 1 had the highest value at 0 hr among the three Treatments. It declined to its lowest value of 170.00 pg/ml at 2-hr post oviposition. From 2 hr onward it started rising towards its peak value of 660.00 pg/ml at 20-hr post oviposition. Between the 2nd and 20th hr, the values of plasma estradiol kept oscillating. Treatment 2 had its minimum value of plasma estradiol at 0-hr post oviposition. Before reaching its peak value of 770.00 pg/ml at 20 hr post oviposition it had several middle peaks of around 600 pg/ml. Treatment 3 had the lowest value at 0 hr among the three Treatments. Except for its peak value of 707.25 pg/ml at 22-hr post oviposition, it was otherwise similar to Treatment 1. The changing

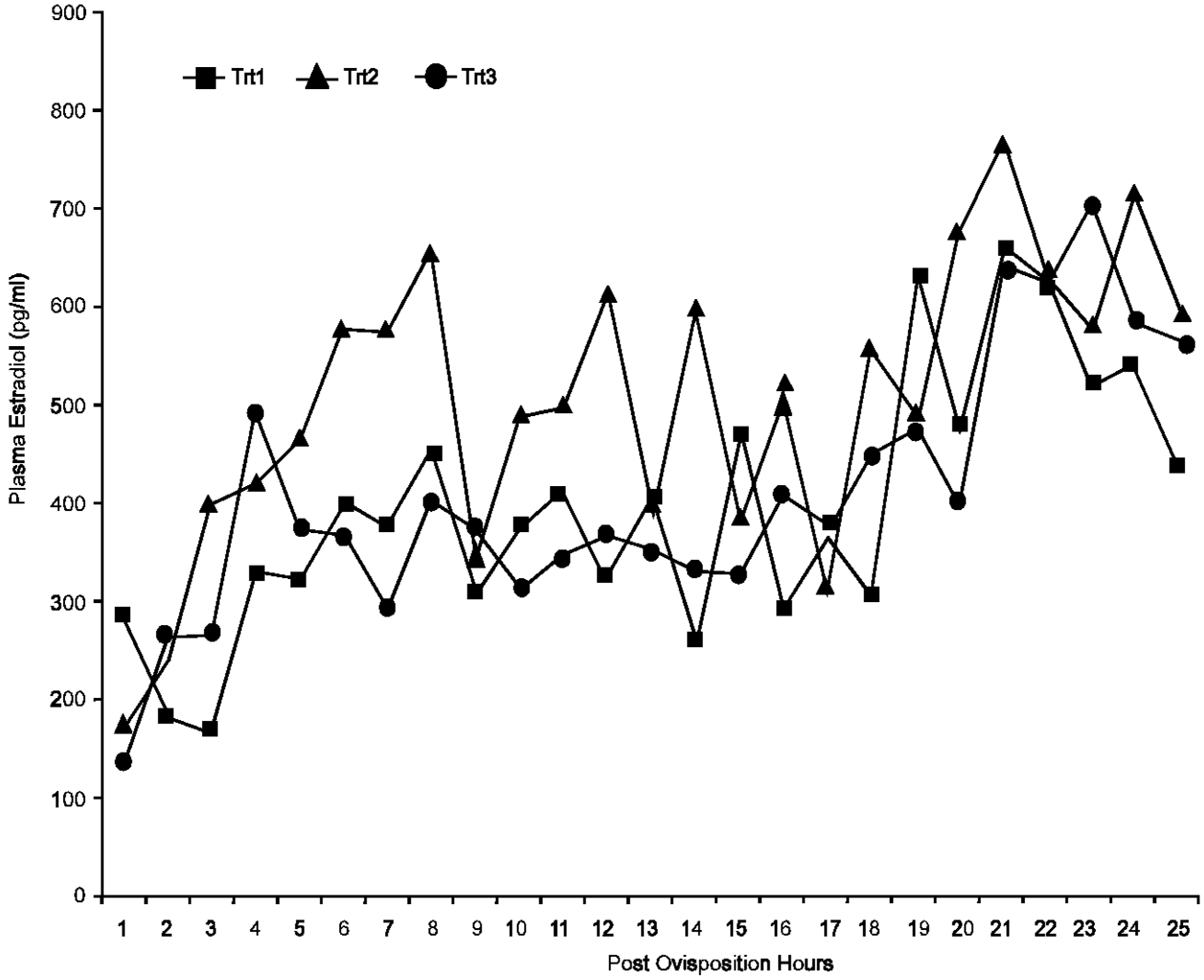

Fig. 3: Mean concentrations of plasma estradiol (pg/ml) at each hour post oviposition for birds fed control (Treatment 1) limestone with reduced phosphorus in the evening feed (Treatment 2) or ½ limestone and ½ oyster-shell with reduced phosphorus in the evening feed (Treatment 3)

patterns of plasma estradiol, during the ovulatory-oviposition cycle, in the present study is generally in agreement with that of Senior and Cunningham (1974) who found peak estradiol concentrations within the 6 hr period immediately preceding ovulation, when blood samples were taken at 4-6 hr intervals for 24 hr. This time approximately matches the 20-hr post oviposition value in the present study (ovulation occurs about 30-75 minutes after the oviposition). The present results are in total disagreement with those found by Shodono *et al.* (1975) who determined the plasma estradiol concentration throughout 36 hr of a regular ovulatory cycle of hens at 1, 2, or 4 hr intervals. The levels varied from 50 pg/ml to 250 pg/ml. Their values were high 24 hr before ovulation, decreased until 10 hr before ovulation, then increased and reached a peak of 4-5 hr before ovulation. These times correspond approximately to 2 hr, 16 hr and 21-22 hr post oviposition and at these times the plasma estradiol concentrations in the present study are opposite. The discrepancies in the results could be due to the methodology adopted by Shodono *et al.* (1975) as they took the blood samples within one particular clutch and at variable sampling times; while the present study consisted of several clutches and only one sampling interval, i.e. 1 hr. The difference in plasma estradiol values might be due to the very sensitive assay employed in the present study compared to 18-year-old analytical techniques. The present results are in agreement with those of Peterson and Common (1972) who reported a peak in peripheral plasma concentrations of estradiol at 18-22 hr and at 2-6 hr prior to ovulation. These times in the present study are 4-8 hr and 20-24 hr post oviposition and correspond to the minimum and maximum peaks observed respectively in the plasma estradiol concentrations among the three Treatments. The results are also comparable to those of

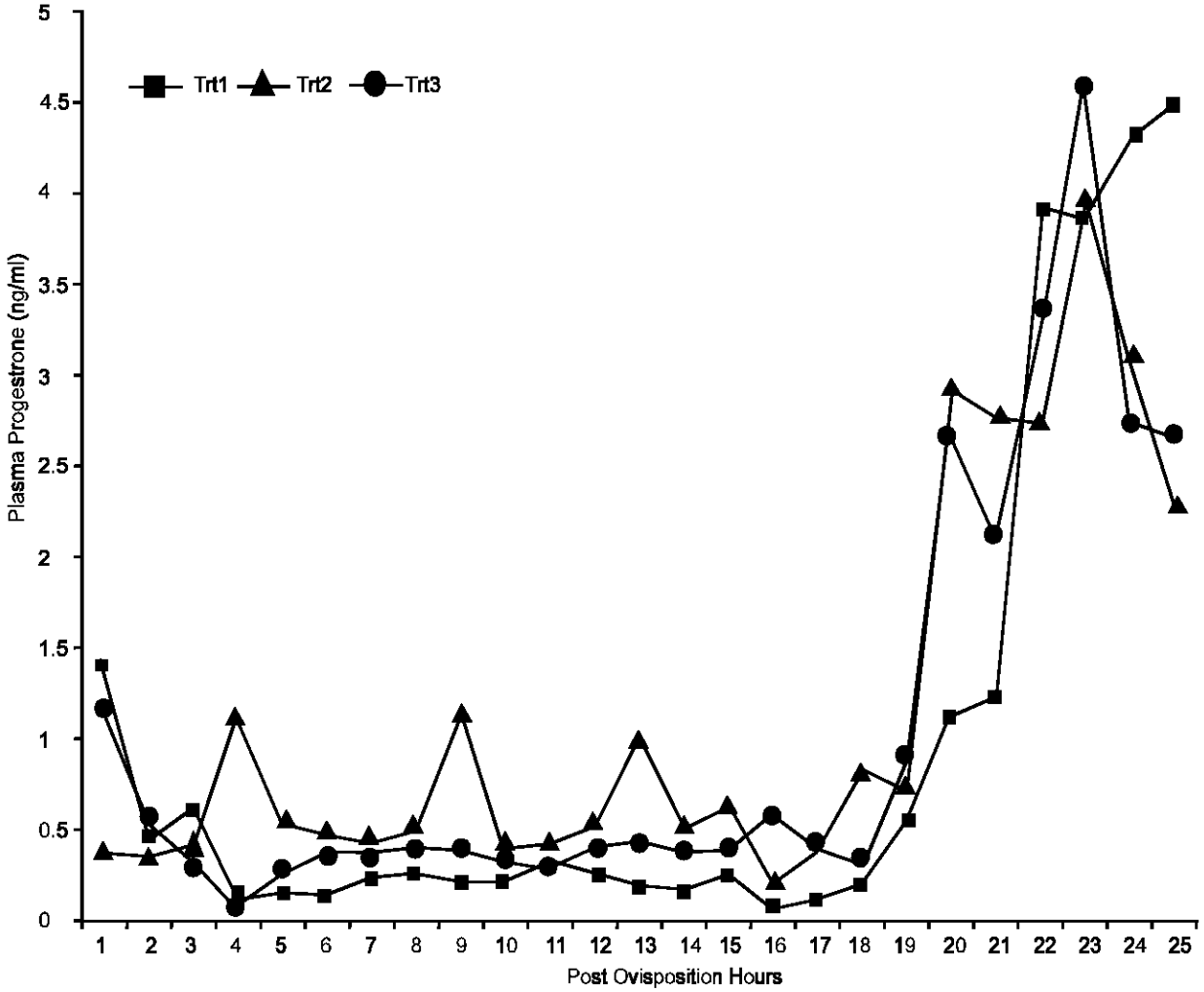

Fig. 4: Mean concentrations of plasma progesterone (ng/ml) at each hour post oviposition for birds fed control (Treatment 1) limestone with reduced phosphors in the evening feed (Treatments 2) or ½ limestone and ½ oyster-shell with reduced phosphors in the evening feed (Treatment 3)

Curl *et al.* (1985) who did not find a high correlation between the shell quality in laying hens and plasma concentrations of estradiol during the 6 hr before ovulation. The corresponding time (20 hr post oviposition) in the present study showed peak plasma estradiol values in all the three Treatments. These values were not different from each other and showed no relationship to eggshell quality. Treatments 3 which otherwise showed better eggshell quality trends by observed parameters (eggs specific gravity and reduced plasma inorganic phosphorus concentrations) had lower plasma estradiol ($P<0.05$) than Treatment 2. It is concluded that plasma estradiol concentrations had no relationship to eggshell quality.

Plasma progesterone: The average plasma progesterone concentrations, at each hour post oviposition among the three Treatments, are presented in Fig. 4. The data obtained were statistically analyzed by factorial analysis of variance using repeat measurements with treatment and time as the main factors. There was a significant difference among treatments, ($P<0.05$), time ($P<0.001$), and the treatment by time interaction ($P<0.01$). Overall treatment means were 1.020, 1.175 and 1.080 ng/ml, respectively, for Treatments 1, 2 and 3. Treatment 2 was different ($P<0.05$) from Treatment 1, but not from Treatment 3, while Treatment 1 and 3 were not different from each other. The one prominent fact among all 3 Treatments was a definitive peak at 22-24-hr post oviposition. All the Treatments then started declining towards the beginning of the next cycle. The three treatments maintained very low plasma progesterone concentration during 1-18 hr post oviposition, except Treatment 2, which had some spikes in between. These results are in full agreement with those of Johnson and Van Tienhoven (1980) who

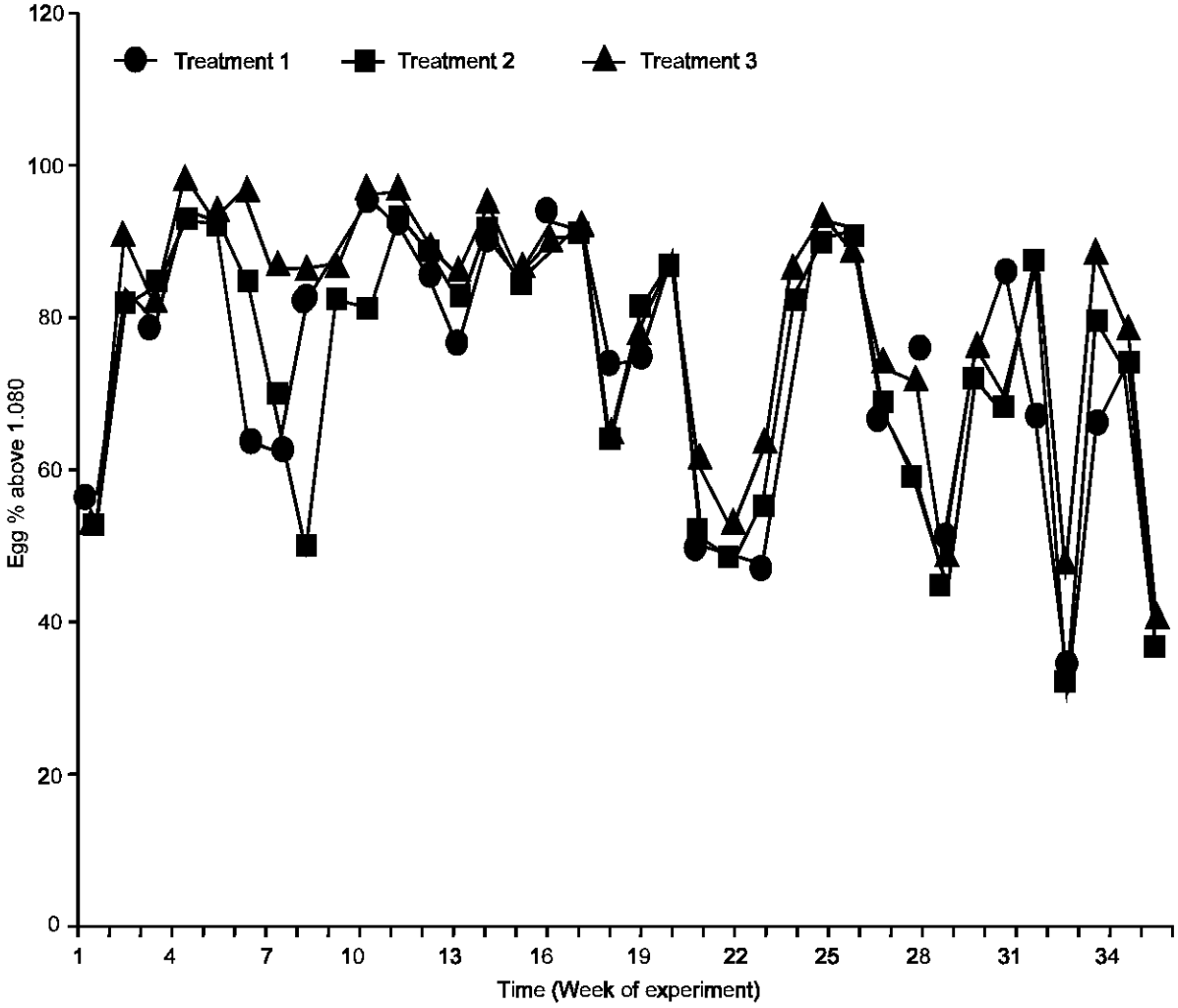

Fig. 5: Mean percentage of eggs with specific gravity above 1.080 by week for birds fed control diet (Treatment 1) limestone with reduced phosphors in the evening feed (Treatment 2) or ½ limestone and ½ oyster shell with reduced phosphorus in the evening feed (Treatment 3)

consistently found peak plasma concentrations of progesterone 6 to 2 hr prior to ovulation when laying hens were serially sampled at 2 hr intervals for a period of 24 to 72 hr. This time is equivalent to 20 to 24 hr post oviposition, when the distinctive peaks in all three Treatments were observed in the present study. These findings, in their broader sense, are also in agreement with those of (Kappauf and Van Tienhoven, 1972; Peterson and Common, 1971; Haynes *et al.*, 1973; Lague *et al.*, 1975; Shodono *et al.*, 1975) who all found plasma progesterone peaks near 2-7 hr prior to ovulation. Shodono *et al.* (1975) further reported that progesterone values varied from 1 ng/ml to 6 ng/ml. The present range was somewhat lower with a range of 0.100 to 4.613 ng/ml. These results confirm the findings of (Lague *et al.*, 1975; Shahabi *et al.*, 1975; Hammond *et al.*, 1980; Johnson and Van Tienhoven, 1980) who found maximum plasma concentrations of both progesterone and estradiol about 3 to 6 hr before ovulation. However, it is hard to match with their conclusion that eggshell quality and estradiol and progesterone concentrations in plasma may be related during the period when hormone concentrations were maximum. As in the present study, Treatment 3, which showed better egg specific gravity than Treatment 2, had lower concentrations of both plasma estradiol and progesterone. The results are similar to those of Curl *et al.* (1985) who found no association between plasma concentrations of estradiol and progesterone and eggshell quality.

Egg specific gravity: The percentages of eggs equal to or above 1.080-egg specific gravity were 74.7, 72.4, and 79.9 for treatments 1, 2, and 3 respectively ($P<0.05$) and are presented in Fig. 5. There was a significant difference among the treatment means ($P<0.05$).

Treatment 3 (control diet in the morning and reduced phosphorus in the evening with ½ limestone and ½ Oyster shell as the calcium source) eggs had higher specific gravities than treatment 1 (control diet, morning and evening) ($P<0.05$). Treatment 3 was also higher than treatment 2 (control diet in the morning and reduced phosphorus with all limestone as calcium source in the evening) ($P<0.05$). Treatment 1 and 2 however, were not different from each other.

The improvement in specific gravity of treatment 3 appears to be a function of the calcium source (Oyster shell), rather than reduced phosphorus. This was evident by the lack of any difference between treatments 1 and 2, where reduced phosphorus was the only difference between the two treatments. These results are in agreement with the previous reports (Moran *et al.*, 1970; Scott *et al.*, 1971; Brister *et al.*, 1981) that partial replacement of limestone with Oyster shell in the diet improved different aspects of shell quality, including egg specific gravity. Results are further substantiated by a report (Keshavarz and Nakajima, 1993) that did not find any significant difference in eggshell quality when available phosphorus was reduced from 0.4 to 0.2% with constant 3.5% calcium in laying hen diets.

It is concluded that Oyster shell or the reduced phosphorus level in the layers' diet did not raise the plasma calcium level in either of the treated groups as compared with the control that had a significantly higher plasma calcium level. Reduced dietary phosphorus decreased plasma inorganic phosphorus in Treatment 3, but this phenomenon may be related to the calcium source. Limestone alone with reduced phosphorus (Treatment 2) did not decrease plasma phosphorus. Reduced dietary phosphorus and/or calcium source appears to have affected blood levels of estradiol and progesterone, but the results are inconsistent among the three Treatments.

Eggshell quality can be improved by partial (½) replacement of the usual calcium source, limestone, with Oyster shell. Although, reduced phosphorus did not directly improve eggshell quality, it might be speculated that it did help indirectly by reducing plasma inorganic phosphorus, the excess of which is otherwise detrimental to eggshell quality.

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