This is to certify that the

dissertation entitled

EGGSHELL QUALITY AND BLOOD PHYSIOLOGICAL PARAMETERS OF LAYING HENS AS INFLUENCED BY ALTERNATING DAILY PHOSPHORUS LEVELS AND DIFFERENT CALCIUM SOURCES.

presented by

Hafiz Anwar Ahmad

has been accepted towards fulfillment
of the requirements for

Ph.D degree in Animal Science

Richard Balander

Major professor

Date 4/8/94

EGGSHELL QUALITY AND BLOOD PHYSIOLOGICAL PARAMETERS OF LAYING HENS AS INFLUENCED BY ALTERNATING DAILY PHOSPHORUS LEVELS AND DIFFERENT CALCIUM SOURCES.

by

Hafiz Anwar Ahmad

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

1994

Richard Balander, Advisor

ABSTRACT

EGGSHELL QUALITY AND BLOOD PHYSIOLOGICAL PARAMETERS OF LAYING HENS AS INFLUENCED BY ALTERNATING DAILY PHOSPHORUS LEVELS AND DIFFERENT CALCIUM SOURCES

by

Hafiz Anwar Ahmad

Fourteen hundred and forty birds were divided into three treatment groups of 480. Each treatment group was housed in a Choretime stair-step, four tier cage system. 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 same 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 levels of 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 1/2 limestone and 1/2 oystershell. The experimental diets were fed for 36 weeks of production. Feed and water were provided ad libitum throughout the period. Light was provided 16 hours per day. Egg production, egg specific gravity, eggshell thickness, and egg weights were recorded throughout the experiment to monitor the treatment effects on these production parameters.

Hen day egg production was significantly different ($P < 0.05$) among treatment means. Treatment 3 was better than treatment 2, but not treatment 1. There was no difference between treatments 1 and 2.

Specific gravity was significantly different ($P < 0.05$) among the treatments. Treatment 3 was better than treatments 1 and 2. Treatment 2 was not different from treatment 1.

The treatment means for eggshell thickness were not significantly different from each other.

Egg weights were measured only twice throughout the experiment. Calcium source and/or reduced phosphorus levels did not affect egg weights. Only advance in age increased the egg weight.

Twenty four birds from each treatment were individually caged for blood sampling. They were housed under the same conditions as those used for the main experiment and were fed the same experimental diets. If a bird laid an egg, the time was noted and a blood sample (3 ml) from that bird, was collected at some hourly interval post oviposition by lateral cardiac puncture. 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 zero to 24 hours were collected from each treatment. Blood was analyzed for plasma calcium, inorganic phosphorus, estradiol and progesterone levels.

There was a significant difference ($P<0.05$) among treatments for plasma calcium. Treatment 1 had higher plasma calcium levels compared to treatments 2 and 3 which were not different from each other.

There was a significant difference ($P<0.01$) among treatments for plasma inorganic phosphorus concentrations. Treatment 3 had a lower concentration of plasma inorganic phosphorus compared to treatments 1 and 2. Treatments 1 and 2, however, were not different from each other.

Plasma estradiol was significantly different ($P < 0.001$) among treatments. Treatment 2 was higher than treatments 1 and 3. Treatments 1 and 3 were not different from each other.

There was a significant difference ($P<0.05$) among treatments for plasma progesterone. Treatment 2 was higher than treatment 1, but not 3, while treatments 1 and 3 were not different from each other.

I dedicate this dissertation to my father, late Rashid Ahmad who always wanted me to be very successful in this world.

ACKNOWLEDGMENTS

I want to thank Almighty Allah Who enabled me to reach this position. I am also highly grateful to Dr. Richard Balander, my major professor for his very understanding attitude, help and dedication towards the completion of my Ph.D.. I wish to thank my committee, Drs. Richard Aulerich, Cal Flegal, Robert Cook and Al Booren for their critical and professional advice throughout the course of this study. I sincerely appreciate the keen interest of the committee in the shaping of this manuscript.

I appreciate the assistance of Angelo Napolitano and all the other people at the MSU Poultry Farm who helped during the course of this experiment.

I want to express my deepest gratitude to my wife, Samina, my daughter, Sana, my mother, brother and sisters for their help and prayers throughout my life.

TABLE OF CONTENTS

LIST OF TABLES

APPENDICES

INTRODUCTION

One of the greatest economic losses to the commercial egg producer results from poor egg shell quality. Roland (1988) surveyed producers representing 81 million commercial layers to determine the percentage of eggs lost or cracked prior to consumer use. Average values obtained for uncollectible eggs, eggs lost at the farm, eggs lost in the processing plant and cracked eggs in the processing plant were 6.1%, 1.6%, 1.16% and 4%, respectively. The percentages of cracked eggs estimated to be in grade A cartons after processing at point of origin and at final destination (store) were 5% and 7%, respectively. Estimates of total eggs cracked or lost prior to reaching their final destination ranged from 13 to 20%, with a dollar loss calculated to be between $1.32 to $2.00/bird/year or a total loss of $477.9 million per year.

There are a number of factors involved in eggshell formation and its subsequent quality. 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 in recent years (Roland, 1986; Rao and Roland, 1989). Oyster shell and limestone are the 2 principal sources of calcium used in laying hen rations. There have been

contradictory reports from earlier experiments indicating that oyster shell was more beneficial than limestone, while others indicated limestone was better than oyster shell for egg shell quality. 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 also important 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, b). 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. 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)

recognized the earlier reports and lowered its recommended total phosphorus level for laying hens from 0.60% (NRC, 1971) to 0.50% (NRC, 1977). The recent 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. Scott et al. (1971) reported that hens became deficient in calcium during the early morning hours. Lennards et al., (1981) reported that the time of calcium intake was important in the ability of the hen to calcify eggshells. 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 et al., 1973). He also 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. Van de Velde et al. (1986) reported peak plasma and organic phosphorus concentration 15 hr after ovulation, which might be attributable to medullary bone resorption during shell formation. It is speculated that this rise in blood phosphorus level interferes, through some mechanisms with calcification of eggshell, on 1 hand and taxes the hen's body through excess excretion on the other. The aim of the present

study was to consider these 3 topics, the calcium source, the phosphorus level and the effect of changing the phosphorus level in the evening feed of the layers, at the same time and to see how these 3 together interact to affect eggshell quality.

HYPOTHESIS

Reduced dietary phosphorus in the evening feed (normal phosphorus in the morning feed) with oyster shell replacing a portion of the limestone as the calcium source will improve the eggshell quality without any skeletal problems in laying hens under the commercial conditions.

LITERATURE REVIEW

EFFECT OF CALCIUM SOURCE ON EGGSHELL QUALITY

The source and particle size of calcium used in laying hen diets are 2 factors that have received considerable attention in recent years (Roland, 1986). Oyster shell and limestone are the 2 principal sources of calcium used in laying hen diets and the effects of feeding these, in both the pulverized or the granulated form, on eggshell quality, have been the subject of many recently published reports. In approximately half of the studies cited in the reviews of Scott et al. (1971), Roland (1986) , and Guinotte (1987), a positive effect of coarse particle size calcium on eggshell quality was reported. These improvements in shell quality were attributed to the particle size rather than the calcium source.

Mongin and Sauveur (1979a) have suggested that the positive effects on eggshell quality of larger calcium particles could be due to the increased intake of calcium just prior to the onset of shell formation. Another suggestion is that the retention of the calcium particles in the crop and their relative insolubility may also make calcium available during the period of shell formation throughout the night when there is no food ingested (Scott et al., 1971; Roland and Harms, 1973; Mongin and Sauveur, 1975). As a consequence of high intake rate and delayed digestion of calcium, the increased amount of available calcium in the

digestive tract during this period would lead to a decreased mobilization of the bone calcium reserve. Such a resorption has been negatively correlated with shell quality (Farmer et al., 1986). Kuhl et al. (1977) indicated that the larger particles allow a release of calcium to the gut at a rate that is optimum for maximum intestinal absorption during eggshell formation.

While comparing oyster shell and limestone and the importance of particle size on eggshell quality, Roland (1986) reviewed 44 papers and concluded that substitution appears to be equally effective in improving shell quality. He further concluded that larger particles of calcium carbonate may only be effective if hens are receiving inadequate calcium or in hens exposed to factors which could reduce the efficiency of calcium utilization (Roland and Harms, 1973; Roland et al., 1974; Bezpa et al., 1974; Muir et al., 1975; Reid and Weber, 1976). Rao and Roland (1989) reported that the percentage of calcium solubilized in the digestive system decreased significantly as calcium intake increased. They also reported significantly increased calcium solubility in the digestive system as the particle size of limestone increased. This suggested a greater retention time of the larger limestone particles in the gizzard. Rabon and Roland (1985) concluded that changing limestone varying in solubility from 8.4% to 34.6% had no short term influence on egg specific gravity.

Calcium carbonate, which is included in the laying hen diet for eggshell formation, is generally obtained from mineral (limestone) or animal deposits (oyster shell). This variation in calcium source leads to a difference in the physical and chemical composition (Reid and Weber, 1976; Charles et al.,

1984). Eggshell weight per unit surface area from hens fed diets containing oyster shell, pulverized eggshell or limestone grit were significantly better than for hens fed diets containing pulverized or ground limestone as the calcium source (March and Amin, 1981; Meyer et al., 1973).

Partial replacement of limestone with oyster shell in the diet has improved different aspects (shell thickness, non destructive deformation, eggshell strength, shell weight, percent shell, and specific gravity) of shell quality as reported by Moran et al. (1970); Scott et al. (1971) and Brister et al. (1981). Makled and Charles (1987) reported that in the absence of $NaHCO_3$ in the feed, specific gravity of eggs was significantly increased by oyster shell (1/3 limestone, 2/3 oyster shell) when the light period was restricted to 16 hr.

Hamilton et al. (1985) conducted 3 experiments on commercial flocks (>16000 birds) through the first and second years of production and evaluated replacing a portion of the dietary calcium from pulverized limestone with hen size particulate limestone or supplementing 3.2% calcium-containing diets with oyster shell. Replacement increased the egg specific gravity without any other improvements in egg quality, while oyster shell supplementation increased the egg production and specific gravity.

Cheng and Coon (1990) concluded through a series of experiments that switching from limestone to oyster shell or switching from highly soluble limestone to a lower soluble limestone and vice versa in short term laying trials showed no significant differences in eggshell quality or layer performance. These include egg weight, egg production and feed conversion. They further

noticed that particle size of the calcium source significantly affected shell weight, shell weight per unit surface area, specific gravity, and shell thickness.

Florescu et al. (1986) could not find any significant difference in number of eggs laid, eggshell thickness, or radiographic appearance of long bones when hens were fed isoenergetic diets with different calcium-sources including 4.5% oyster shell meal. Guinotte and Nys (1991) found higher feed consumption and body weight in hens fed large sized calcium particles (limestone, oyster shell or seashells). Hens fed the ground limestone and particulate oyster shell had smaller egg and shell weights than those fed particulate limestone. Eggs from hens given pulverized limestone in the diet had lower breaking strength than those from hens supplied with particulate limestone. Keshavarz and McCormick (1991) replaced 50% of pulverized limestone with oyster shell as the calcium source in laying hens diets during the summer months. This replacement had a positive effect on egg specific gravity without any improvement on shell strength.

BLOOD CALCIUM

The change in blood plasma or serum characteristics during egg formation has been well documented (Simkiss, 1961; Hodges, 1970). The process of shell formation would utilize all the plasma calcium of a laying hen within 9 to 18 min (Boelkins et al., 1973). To compensate for this depletion, the hen's intestine has a high calcium absorbing capacity (Hurwitz and Bar, 1965; Wasserman and Combs, 1978). Furthermore, laying hens possess medullary bone (in the cavities of the long bones), which can be reabsorbed whenever the rate of

calcium absorption from the intestine is insufficient for eggshell calcification (Mueller et al., 1964).

Hertelendy and Taylor (1961) and Roland et al. (1973) have shown serum calcium to be related to shell quality and quantity when hens were fed a calcium deficient diet. However, Sloan et al. (1974) found no relationship between serum calcium and egg specific gravity when hens were fed adequate calcium diets. Because of variations in egg size, the amount of shell deposited around the egg may or may not have any relationship to the quality of the shell produced (Roland, 1979). The quantity of shell deposited on the egg varies from 3 to 7 g, and the average serum calcium level among producing hens varies from approximately 20 to 30 mg/dl (Taylor, 1970).

Because the shell consists of approximately 40% calcium (60% carbonate), and all calcium reaches the shell gland via blood, it is likely that some relationship exists between serum calcium level and the quality of shell deposited. This relationship could not be confirmed by Lennards et al. (1981) who found no correlation between serum calcium and shell weight or egg weight. However, serum calcium was positively correlated to egg specific gravity in 1 of the 3 experiments conducted. They concluded that the normal variation in serum calcium is not related to the hen's ability to produce eggshell.

Bolden and Jensen (1985) conducted 3 experiments to examine the effect of dietary calcium on plasma calcium, and eggshell quality. Plasma calcium (both diffusible and non diffusible) in the hens fed a corn-soy diet was significantly higher than for hens fed more complex diets. It was also elevated by the higher

dietary calcium in only 1 of the 3 experiments. No significant differences were observed for tibial ash, plasma estradiol, plasma phosphorus, or egg weight.

Van de Velde et al. (1986) observed a drop in plasma calcium concentration from 32.0 to 23.3 mg/100 ml plasma during egg formation. This phenomenon was more of an individual variation. Addition of oyster shell in the diet had no effect on plasma calcium concentrations. Holtea et al. (1985) fed hens 3 diets containing 2% calcium or 3.25% calcium in the morning and 4.25% calcium in the evening feed from granular limestone sources and compared the results to a diet containing 3.5% calcium from oyster shell as the control. The serum calcium was increased by 1.99 to 2.56%. Cheng and Coon (1990) observed no change in blood calcium, when switching from limestone to oyster shell or switching higher soluble limestone to a lower soluble limestone and vice versa in short term laying trials. Guinotte and Nys (1991) concluded that feeding hens diets containing calcium either from particulate or pulverized limestone or oyster shell had no effect on blood pH, plasma ionized calcium, or plasma total calcium.

PHOSPHORUS LEVEL IN LAYER DIETS

Phosphorus is an important element for eggshell formation, not because eggshell contains much phosphorus (there is about 100 times as much calcium as phosphorus in eggshell), but because of the special relationship between calcium and phosphorus in bone formation. Calcium is stored in the skeleton almost entirely as calcium phosphate and therefore synthesis of medullary bone

requires dietary phosphorus. This phosphorus, however, is involved in an essentially futile process because if the calcium from medullary bone 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; and Harms et al., 1982a).

During 1970's the phosphorus requirement of laying hens had received increased attention. Despite considerable research on the subject (Edwards, 1978; Garlich, 1979; Sell, 1979), the optimal phosphorus requirement is still uncertain. It is evident that the phosphorus requirement has been decreasing over the past 30 years. In 1960 the NRC implied that the available phosphorus requirement was 429 mg/hen/day versus 350 mg/hen/day in the 1984 edition. This would be calculating plant phosphorus as 30% available. A problem in determining the phosphorus requirement is that all plant or organic phosphorus might not be totally available. It has been reported that the availability of phytate phosphorus varies from 0 to 100% (Gillis et al., 1953; Harms et al., 1961; Nott et al., 1967; Hamilton and Sibbald, 1977). NRC (1977) states that only 30% of phosphorus in plant products is available to young chicks. However, the older bird has the ability to use most, if not all, of the phytin or organic phosphorus in plants. The quantity of phytate phosphorus in different plants varies, but on average approximately 70% of the plant phosphorus is in the form of phytate.

DIETARY PHOSPHORUS FOR LAYERS

The minimum phosphorus requirement to maintain production is uncertain, but several workers have reported the phosphorus requirement for maximum production is very low, 314 to 440 mg available phosphorous/hen/day. In studies conducted by Roland (1986) total phosphorus intakes of 384 and 307 mg/hen/day maintained production in 2 experiments, but phosphorus intakes of 310, 329, 322 and 288 mg/hen/day did not in 2 other experiments. Van de Velde et al. (1986) showed that the total plasma phosphorus concentration steadily decreased during egg formation from 59.2 to 47.0 mg/dl. Individual variation in the total plasma phosphorus concentration was large and significant. Even though all low phosphorus treatments significantly reduced serum phosphorus, which would supposedly enhance bone resorption, there was little indication that egg specific gravity was improved. Others have reported small improvements in egg specific gravity by reducing phosphorus intake from 677 to 508 mg phosphorus/hen/day (Reichmann and Connor, 1977) and from 730 to 404 mg phosphorus/hen/day (Miles et al., 1983) without reducing serum phosphorus. This suggests that bone mobilization of calcium by reduced phosphorus levels may not explain the occasional slight response reported by some research workers. Roland and Harms (1976); Hamilton and Sibbald (1977); Ousterhout (1980); and Miles et al. (1983) have reported that reducing dietary phosphorus levels below 600 to 700 mg/hen/day gave small improvements in shell quality (egg specific gravity) whereas, Salman et al. (1969); Hunt and Chencey (1970);

Owings et al. (1977); Reichmann and Connor (1977) El Boushy (1979); and Mikaelian and Sell (1981) have reported that reducing dietary phosphorus had no significant effect or had an adverse effect on shell quality. Conversely Taylor (1965); El Boushy (1979); and Miles et al. (1983) have reported that increasing phosphorus above levels typically fed reduces shell quality. Several hypotheses have been offered for the beneficial effect of reducing phosphorus levels on shell quality. The one most accepted is the low phosphorus bone resorption hypothesis (Paul and Snetsinger, 1969; Roland and Harms, 1976; Mongin and Sauveur, 1979b). This hypothesis is based on the belief that during late hours of the night, the hen becomes dietary calcium deficient, which stimulates mobilization of skeletal calcium. As the calcium is withdrawn from the skeleton, so is phosphorus, thereby increasing serum phosphorus (Paul and Snetsinger, 1969), which may have an adverse effect on further bone resorption. Feeding low levels of dietary phosphorus is believed to reduce serum phosphorus and allow for maximum utilization of skeletal calcium as the blood level of phosphorus is directly associated with the dietary level of the element (Hurwitz and Griminger, 1962). However, this hypothesis has been questioned. There are reports which indicate that high dietary levels of phosphorus also increase bone resorption (Draper et al., 1972). Similar patterns of serum phosphorus and calcium in laying hens thought to be associated with shell calcification have been reported in rats (Talmage et al., 1975). Also, high levels of phosphorus (1 to 1.5%) are used routinely in layer diets in some countries when high levels of animal by-products are used.

Another explanation attributed the improved shell quality to a reduction in the formation of insoluble calcium phosphate complexes in the gut (Taylor, 1965). A third possible explanation suggested by Hunt and Chencey (1970) was that phosphorus acts as a crystal poison in the shell gland, thereby inhibiting the growth rate of calcite in the eggshell.

In hens producing large numbers of eggs, Antillon (1976) showed that when dietary available phosphorus was reduced from 5.5 to 2.5 g/kg, egg production was as high as that with the control diet and the breaking strength of the eggs of the hens receiving 2.5 g/kg available phosphorus was significantly superior to that of those receiving 5.5 g/kg. A reduction in eggshell quality from the inclusion of dietary phosphorus in excess of that required for maximum egg production has also been reported by Damron et al. (1974), Bletner and McChee (1975), Summers et al. (1976), Harms and Miles (1980) and Guenter (1980). Hopkins, et al. (1987) conducted 2 studies with Shaver and ISA brown birds. In the first study the egg shell density was maintained at the highest levels with diets providing 0.23 g/day of non-phytin phosphorus. A daily intake of 4.1 g calcium and no more than 0.55 g total phosphorus containing 0.23 g non-phytin phosphorus was found to produce optimum eggshell quality. At higher phosphorus intakes, more calcium was needed to maintain shell quality. In the second study 5.3 g of calcium and 0.63 g total phosphorus containing 0.20 g non-phytin phosphorus produced optimum eggshell quality. Eggshell density was reduced at higher dietary phosphorus and did not improve with higher calcium intakes. Day, et al. (1987) found significantly higher egg production and

egg breaking strength for hens given 0.31% total phosphorus as compared to those who were given 0.36 to 0.61% total phosphorus. Perez and Osa (1987) concluded that available levels of phosphorus and calcium could be reduced to 0.40% and 3.00%, respectively, of the diet without disturbing the production performance of hens. Keshavarz (1986) found no significant difference in egg production, egg weight, feed consumption, plasma calcium and phosphorus, egg shell quality, and tibia ash when hens were fed 3.5, 4.5 and 5.5% calcium and 0.24, 0.44 and 0.46% available phosphorus. The only exception was significantly lower feed consumption and plasma phosphorus level with the lowest dietary phosphorus level. Roush et al. (1986) used response surface methodology to examine the effect of calcium and available phosphorus on biological responses associated with egg production. In their study, optimal levels of calcium and available phosphorus for the percent eggshell were calculated to be 4.73% and 0.48%, respectively. Daghir et al. (1985) concluded that laying hens required a minimum of 0.25% available phosphorus for best egg production, feed consumption and body weight gain. Available phosphorus levels above 0.35% depressed shell thickness.

Hartel (1989) used a wide range of calcium contents (20 to 45 g/kg) to investigate responses to changing dietary phosphorus (3.2 to 16.2 g/kg) when dietary calcium varies and vice versa. He concluded that the maximum total phosphorus requirement for the laying hen was either 3.2 g/kg diet or 360 mg/hen/day when the calcium concentration was restricted to 25 g/kg. However, to achieve the highest egg production and lowest mortality 7.0 to 8.0 g/kg or 880

- 1020 mg/hen/day total phosphorus were needed. To keep the percentage of damaged or shell-less eggs low at the same time the calcium content of the food should be 30 g/kg or less. Rao et al. (1992) used early maturing (EM) and late maturing (LM) pullets to observe the effects of low dietary phosphorus (0.4% total phosphorus) and normal dietary phosphorus (0.7% total phosphorus). Low phosphorus caused severe adverse effects on late maturing pullets. The LM pullets fed the low phosphorus diet had high plasma ionic calcium concentration, low plasma inorganic phosphorus concentration, increased urine calcium concentration, a high incidence of osteoporosis, mild kidney lesions, and elevated mortality compared with pullets subjected to other treatments. The EM pullets fed the low phosphorus diet were also adversely affected by low phosphorus, but were less susceptible to osteoporosis and mortality. The low phosphorus diet improved eggshell quality, but this beneficial effect was only temporary. The severity of adverse effects of low dietary phosphorus was greater for LM than the EM pullets. Vandepopuliere and Lyons (1992) conducted 2 experiments to observe the effects of different inorganic phosphorus sources with variable levels on laying hen performance and eggshell quality. In experiment 1, a regular and a coarse form of defluorinated phosphate and one source of dicalcium phosphate were used in diets containing either 0.4 or 0.5% total phosphorus (0.2 or 0.3% nonphytate phosphorus). In experiment 2, either the regular form of defluorinated phosphate or the dicalcium phosphate source was fed in diets that contained either 0.4, 0.5, 0.6 or 0.7 % total phosphorus (0.2, 0.3, 0.4, or 0.5 % nonphytate phosphorus). In experiment

1, hens fed 0.5% total dietary phosphorus consumed more feed and produced heavier eggs. No differences among dietary treatments existed for egg specific gravity. A significant source by level interaction occurred for the farm classification of thin-shelled, cracked or broken eggs and for the total of these classifications. In experiment 2, egg production, egg weight, and egg mass were depressed at the 0.4% total dietary phosphorus level. Hens fed 0.4 and 0.7% total dietary phosphorus levels laid eggs with the highest and lowest egg specific gravity, respectively. This trend was inverse to the effect of these phosphorus levels on egg weights. At the 0.4% total phosphorus level, the chickens consuming the regular form of defluorinated phosphate produced eggs with a significantly thinner shells than the birds consuming the dicalcium phosphate source. Hens fed the dicalcium phosphate source produced a higher percentage of compressed-sided and misshapen eggs. In a recent long-term experiment, Keshavarz and Nakajima (1993) found no significant difference in eggshell quality when available phosphorus was reduced from 0.4 to 0.2% with constant 3.5% calcium in laying hen's diets.

BLOOD PHOSPHORUS

In hens receiving a conventional diet with ground limestone, plasma phosphorus starts increasing prior to the dark period, reaches a maximum during the dark period and then starts declining to its lower concentration by the end of the dark period (Miller et al., 1977a and Mongin and Sauveur 1979b).

The latter authors emphasized that the increase in phosphate concentration before the onset of the dark period is not simply by skeletal mobilization during the dark period, but by increased intestinal phosphate absorption. They also showed that if a persistent source of calcium (seashell grit) was made available to hens, the response in plasma phosphorus was modified. Van de Velde et al. (1986) reported that the total plasma phosphorus concentration steadily decreased during egg formation from 59.2 to 47.0 mg/100 ml. Individual variation in the total plasma phosphorus concentration was large and significant. The inorganic plasma phosphorus concentration reached a peak of 6.9 mg/100 ml at 15 hr after ovulation and fell to 6.0 mg/100 ml at 22 hr after ovulation.

Arscott et al. (1962); Taylor (1965) and Harms (1982a) have reported that dietary excess of phosphorus has a detrimental effect on shell quality. They could not explain 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 the blood phosphorus on the shell forming process. Miles and Harms (1982) showed a clear linear negative correlation between specific gravity and plasma phosphate over a range of treatments.

Common (1932, 1933, 1936) reported a rise in the phosphorus content of the excreta corresponding to the period of shell formation and attributed the excess phosphorus to that set free when the calcium was liberated from lime for shell formation. Feinberg et al. (1937) was first to report that the serum of laying hens shows a marked rise in inorganic phosphorus during the period of egg shell

formation. The amount of inorganic phosphorus in the serum increased from about 4.5 to 7.5 mg/dl between oviposition and the first hours of shell calcification (11.5 to 16 hr post oviposition of the previous egg). The increase in the serum phosphorus was ascribed to the liberation of inorganic phosphorus from bone when calcium was liberated for shell calcification purposes. According to Paul and Snetsinger (1969) and Speers et al. (1970), there was no increase in serum inorganic phosphorus just before the beginning of egg shell formation (9 hr post oviposition). These authors reported a significant increase in phosphorus at 16 hr post oviposition that disappeared by 23 hr post oviposition. Miller et al. (1977 a, b) and Mongin and Sauveur (1979b) have confirmed the cyclic nature of the serum phosphorus in relation to the daily egg laying cycle. They have further confirmed that the inorganic phosphorus level in serum decreases sharply 2 to 3 hr prior to oviposition. Miller et al. (1977a) reported only a slow gradual increase in serum phosphorus during shell formation while Mongin and Sauveur (1979b) reported a rapid increase beginning approximately 10 hr post oviposition. In these studies, peaks in phosphorus were reported at approximately the same time during the daily egg laying cycle (approximately 22 hr post oviposition of the previous egg). Miles et al. (1983) conducted 2 experiments in which blood samples were collected and plasma phosphorus was determined immediately following oviposition (0 hr) or 6 to 21 hr post oviposition from hens laying in the morning or afternoon. In the first experiment, plasma phosphorus at oviposition was the same for hens laying in the morning or afternoon (3.95 and 4.10 mg/dl, respectively). At 6 hr post

oviposition plasma phosphorus was lower than that observed at 0 hr (3.22 and 3.15 mg/dl, respectively). When plasma phosphorus levels at 21 hr post oviposition of hens laying in the morning or afternoon were compared, a higher phosphorus level was observed for hens that laid in the morning (5.90 vs. 4.91 mg/dl, respectively). In the second experiment, plasma phosphorus levels at 0 and 6 hr post oviposition were 3.32 vs. 3.18 and 2.81 vs. 2.74 mg/dl for hens laying in the morning and afternoon, respectively. At 21 hr post oviposition, phosphorus values of 5.30 vs. 3.65 mg/dl were reported for morning and afternoon laying hens. Plasma phosphorus did not rise on the day when a hen did not lay an egg. In another trial Keshavarz (1986) compared 3 calcium (3.5, 4.5 and 5.5 %) and 3 available phosphorus (0.24, 0.44 and 0.64%) levels in the diets of 56 wk old commercial laying hens for 16 weeks. Plasma calcium and phosphorus and egg shell quality were not significantly affected by dietary levels of phosphorus except the lowest dietary phosphorus had a lower plasma phosphorus.

Ruschkowski and Hart (1992) compared the inorganic plasma phosphorus among control (standard corn-soy diet), calcium deficient and vitamin D deficient diets. Plasma concentrations of inorganic phosphorus were cyclic in nature in the control hens. Inorganic phosphorus concentration increased during the light period of the day, then decreased during the dark phase in vitamin D deficient hens. There was no change in inorganic phosphorus concentration in the calcium deficient hens during the 24-hr period. Mean plasma inorganic phosphorus concentrations were depressed in the calcium-deficient (3.28 mg/dl)

and to an even greater extent in the vitamin D deficient hens (1.50 mg/dl) in comparison with the control hens (7.34 mg/dl).

Sooncharernying and Edwards (1989) fed laying hens a diet containing combinations of 2.6 or 3.6% calcium and 0.45 or 0.75% total phosphorus. Blood samples were collected during a 24-hr period at 6-hr intervals and total calcium, ultrafilterable calcium and dialyzable phosphorus were determined. Plasma dialyzable phosphorus was significantly influenced by time increasing from 2.67 mg/dl at 0600 hr to 3.48 mg/dl at 1800 hr and then declining to 2.74 mg/dl at 2400 hr. The dialyzable phosphorus level was not affected by dietary phosphorus level. The authors speculated the results are from increasing bone demineralization and possibly due to the inability of the kidneys to excrete phosphorus at a rate equal to that of its mobilization from bones during this period.

Frost and Roland (1991) fed 3 dietary levels of total phosphorus, 0.30, 0.60 or 0.90% to 75 wk old hens. Blood samples were collected at 6 different times, 2, 6, 10, 14, 18 and 22 hr post oviposition and analyzed for phosphorus. Plasma phosphorus significantly decreased with decreasing dietary phosphorus. Plasma phosphorus peaked at 16 hr post oviposition.

CAGE LAYER FATIGUE

Cage layer fatigue is the most significant skeletal disease of the caged layer in production (American Association of Avian Pathologists, 1987). Cage layer fatigue is also considered the primary cause of fractures and breakage at the

processing plants in spent fowls (Riddell, 1989). In the mid fifties the term cage layer fatigue was first used by Couch (1955) to describe a combination of leg weakness and acute deaths. The demands to produce eggs in layers put a tremendous pressure on the calcium homeostatic mechanisms of the birds. The laying hens puts 2.3 g of calcium into each eggshell and another 25 mg into the yolk (Etches, 1987). Over a 70 wk period of production, the hen may utilize more than 580 g of calcium for eggshell formation.

Calcium is transferred from blood to the eggshell at an approximate rate of 100-150 mg/hr (Soares, 1984). The plasma calcium would be reduced to zero within 15-30 min if alternative sources of calcium were not available (Etches, 1987). A specialized woven bone (medullary bone) formed under the influence of estrogen is presumed to provide the necessary calcium to maintain calcium homeostasis (Simkiss, 1967). It is estimated that 30-40% of the eggshell calcium comes from bones (Mueller et al., 1964). This presumption is based upon several general observations; the turnover rate of medullary bones is twice that of cortical bones (Hurwitz, 1965), medullary bones are maintained at the expense of cortical bones during the periods of calcium deprivation (Simkiss, 1967) and the mass of medullary bone is greatest immediately prior to the onset of eggshell calcification (Candlish, 1981). In addition, the osteoclast number and resorption surface increase in medullary bones during eggshell calcification (Miller, 1981) and acid phosphatase activity is increased in the plasma (Taylor et al., 1965).

The etiology of the cage layer fatigue has been considered to be primarily nutritional (Riddell et al., 1968) although both strain and housing of the birds may affect the incidence (Francis, 1957). Frequently, birds at their peak production are reported to develop this problem although they have a negative calcium balance once they start their production (Hurwitz, 1965). The lesions have been described as an osteoporosis of the cortical bone (Bell and Siller, 1962) or osteomalacia involving the non-weight bearing medullary bones (Antillon et al., 1977).

ESTRADIOL

The hen lays eggs in sequences or clutches, a clutch being the number of eggs laid on consecutive days. The clutch ends with a day on which no egg is laid. The pattern of ovulation also follows that of egg-laying or oviposition, the time interval from ovulation to oviposition being around 24-28 hr. Oviposition of one egg of the clutch is followed approximately 30 min later (Warren and Scott, 1935) by ovulation of the next follicle, except for the last oviposition of the clutch. The most attractive theory that has been proposed to explain these events in that of Fraps (1955a, 1961) who postulated that rising plasma concentrations of an "excitation hormone", secreted by the maturing ovarian follicle, causes the release of luteinizing hormone by acting on the hypothalamus, and that the sensitivity of the neural component to the "excitation hormone" varies on a 24 hr circadian basis. The failure to ovulate which ends the clutch of eggs occurs when the rising concentration of "excitation hormone" coincides with a time of

hypothalamic insensitivity. On the basis of experiments involving the injection of steroids (Fraps, 1955 b), the disruption of the hypothalamic tracts (Ralph and Fraps, 1959) and the direct injection of progesterone into the hypothalamus Ralph and Fraps, (1959) claimed that the "excitation hormone" was progesterone. Senior and Cunningham (1974), through radioimmunoassay, suggested that estradiol may be involved in the mechanism stimulating the release of luteinizing hormone required for ovulation. During the ovulatory cycle when blood samples were taken at 4 or 6 hr intervals for 24 hr, the peak concentrations of estrone and estradiol occurred within the 6 hr period immediately preceding ovulation. They also found no significant change in estradiol concentration over the 24 hr period in non laying hens. Estradiol concentration rose 8 hr before ovulation and reached a peak level 4-6 hr before ovulation, when blood samples were taken at 2 hr intervals during the 12-14 hr period. Shodono et al. (1975) determined the plasma estradiol concentration throughout 36 hr of regular ovulatory cycles of hens, starting 24-25 hr before the second egg (C_2) ovulation of the clutch and ending 11-12 hr after C_2 ovulation, at 1, 2 or 4 hr intervals. The level varied from 50 pg/ml to 250 pg/ml. The value was high 24 hr before ovulation, decreased until 10 hr before ovulation, then increased and reached a peak 4-5 hr before ovulation, when it started to decrease again until 12 hr after ovulation. Peterson and Common (1972), however, reported a peak in peripheral plasma concentrations of estradiol at 18-22 hr and at 2-6 hr prior to ovulation. Lague et al. (1975) found that the estrogen peaks precede ovulation by 4-7 hr. In their study, ovulation did occur

occasionally in the absence of estrogen peaks and some estrogen peaks appear not to be related to ovulation. Johnson and van Tienhoven(1980) found the highest concentration of estradiol -17β and estrone 6 hr prior to the ovulation when hens were serially sampled at 2 hr intervals for a period of 34 to 72 hr. Kamar et al. (1982) obtained blood samples from 100 hens at 25 intervals (intervals not defined in paper) before laying the 1st egg, and at intervals during the laying cycle. Estradiol-17β concentration increased from 9.0 pg/ml 42 days before the 1st egg to 77 pg/ml 2 days before the 1st egg. During the laying cycle, estradiol concentrations showed peaks (240.0 ± 50.0 to 275.0 ± 50.0 pg/ml) 3-4 and 21-23 hr before oviposition, the basal concentration was 35-50 pg/ml.

In general, there is agreement that at least 2 peaks of estradiol precede an ovulation, one occurring 20 to 16 hr and one 7 to 2 hr prior to ovulation; during a night preceding a day when no ovulation occurs, there is no significant fluctuation of estradiol (Peterson and Common, 1971; Senior, 1974). The role of these estradiol peaks with respect to ovulation is not clear. Injection of estradiol 14 to 12 hr prior to ovulation of the C_1 follicle does not induce premature ovulation and fails to demonstrate an additive or synergistic effect with progesterone to induce premature ovulation (Lague et al., 1975).

Estrogen has been reported to be involved in medullary bone formation (Beniot and Calvert, 1945). In the hen, mean serum concentrations of estradiol -17 β begin to rise sharply 17 weeks before laying is initiated, reach a peak 2-3 weeks before the onset of egg production, and then drop to medium levels when

egg lay is established. The high serum levels of estradiol-17 β found before the onset of egg-lay may be essential for the synthesis of yolk and medullary bone in preparation for eggshell formation. The effects of exogenous estrogen on calcium metabolism in birds are well documented. Kaetzel and Soares (1984) increased total plasma calcium, bone-breaking strength, and bone ash in quail hens with estradiol-17 β implants. Lobaugh et al. (1981) observed increased plasma calcium after 12 days of I.M. injections of 2 mg/kg estradiol diproprionate. McMurty and Forbish (1981) reported that both total and ionized blood calcium were increased after 2 injections of 25 mg of estrogen, while only total serum calcium was increased by 2 injections of 75 mg of estrogen. Estrogen administration also has been shown to affect egg production. Grunder et al. (1980) reported erratic production following injection of 10 mg estradiol-17 β benzoate, with hens going out of production after laying 3-4 eggs. Douglas et al. (1978) showed the onset of production to be delayed 2-3 weeks in hens fed 160 mg dienestrol diacetate (a synthetic estrogen) per pound of diet; in addition, egg production was reduced, but egg weight increased.

Taher et al. (1986) conducted 2 experiments on the effects of long-term, low levels of exogenous estradiol-17β and dietary calcium on calcium metabolism and laying hen performance. Their results suggested no increase in calcium utilization by estradiol -17β at very low dietary calcium. They further concluded that egg production, egg weight and eggshell strength are important indicators of endocrine stability in the hen. Tsang and Grunder (1985) observed a significant difference in plasma estradiol between 2 strains of hens laying eggs with

different egg shell quality, but with the same egg production and egg weight. The estradiol concentrations in the 2 strains at 118 days were 108 and 98 pg/ml and at 146 days were 261 and 159 pg/ml, respectively.

Exogenous estrogen induces or stimulates hepatic synthesis of certain calcium-binding proteins (Urist et al., 1958) of which vitellogenin is a major one (Guyer et al., 1980). The increased release of the calcium binding proteins then result in elevated protein-bound calcium in the plasma. Diethylstilbestrol (DES), which is a non-steroid estrogen, has been shown to stimulate differentiation of the shell gland of 7.5 to 10 wk old chicks and to induce the formation of a calcium binding protein (Navickis et al., 1979). Indirect evidence linking estrogen to calcification of the egg comes from generic studies in which a line of chickens selected for thick shells had higher levels of total serum calcium than a line selected for thin shells (Clagett et al., 1977). After estradiol administration, the thick shell hens and roosters had higher levels of serum calcium binding as well as higher levels of vitellogenin (Grunder et al., 1978, 1980). Calcium homeostasis is achieved by balancing the efficiency of intestinal calcium absorption, renal calcium excretion, and bone mineral metabolism to the animal's calcium requirements. The main hormones controlling this balance are parathyroid hormone and 1,25-dihydroxy vitamin D_3 [1,25-$(OH)_2$ D_3] produced by the renal conversion of 25-hydroxyvitamin D_3 (25-OHD_3) through the activity of the enzyme 25-OHD_3-1α-hydroxylase (Lawson et al., 1971; Kenny, 1976; Swaminathan et al., 1977). In growing or egg producing birds, the calcium demands are high and are associated with high renal production of 1,25-$(OH)_2D$ (Sommerville et al., 1983; Abe et al., 1979). In the chick, 1,25-$(OH)_2D$ accumulates in the intestinal mucosa, increasing the rate of calcium absorption (Sommerville et al., 1978). It has been shown that high levels of estrogen may

affect the metabolism of 1,25-$(OH)_2$ D_3, but it is unclear whether this is a direct effect on 1-hydroxylase activity or is secondary to increasing calcium demands (Kenny, 1976; Castillo et al., 1977) or is an indirect effect of increases in circulating growth hormone and prolactin (Spanos et al., 1978; Sommerville et al., 1983; Scanes et al., 1984). In the laying hen high levels of circulating calcium bound to plasma protein occur and the renal 1-hydroxylase activity shows cyclic fluctuations related to oviposition (Abe et al., 1979). It has recently been shown (Tsang et al., 1988) that laying hens deficient in vitamin D or calcium have a reduced rate of metabolic clearance of estrogen, again demonstrating the interrelation between estrogen, calcium and vitamin D metabolism. This reduced rate of estrogen clearance developed a metabolic defect in the conversion of estradiol-17β-3-sulphate to estradiol-17α-3-sulphate which resulted in an accumulation of the former and a reduced formation of the latter (Tsang and Grunder, 1985). This was the first evidence that calciferol directly or indirectly affected estrogen catabolism. Ruschkowski and Hart (1992) compared the plasma concentrations of total calcium, ionic calcium, inorganic phosphorus, estradiol-17β, progesterone, and 1, 25-dihydroxycholecalciferol during a single ovulatory cycle in calcium-deficient and vitamin D-deficient hens with those of normal hens. Mean estradiol-17β concentrations were depressed in both groups of deficient hens (calcium-deficient, 67 pg/ml; vitamin D-deficient, 43 pg/ml) compared with the control hens (99 pg/ml). It is hard to pin point the precise physiological role of the circulating estrogen in relation to ovulation, but it is conceivable that estrogen may influence egg shell quality by elevating protein bound calcium in plasma and enhancing calcium transport in the shell gland. Grunder et al. (1983) compared the plasma levels of estrogen and its conjugates and calcium between the 2 strains of hens laying eggs with different shell quality (high and low egg specific gravity). They concluded that the strain

with better shell quality had significantly greater levels of plasma estrone, estrone sulfate, and estradiol, but not estradiol sulfate or total calcium, than the strain with lower shell quality at 6 to 8 hr. post oviposition, suggesting a relationship between plasma hormone levels and shell quality. Curl et al. (1985), however, could not produce a high correlation between the shell quality in laying hens and plasma concentrations of estradiol during the 6 hr before ovulation. They also found no significant difference in egg production and egg weight between the shell quality groups.

PROGESTERONE

Several steroids have been implicated in the induction of ovulation in the domestic hen, including progesterone (Wilson and Sharp, 1975; Etches and Cunningham, 1976). Quantification of hormone concentrations in serial blood samples taken throughout the ovulatory cycle have resulted in numerous reports of cycle-related rhythms of various combinations of 2 or 3 steroids and leutinizing hormone. Despite the findings that plasma concentrations of progesterone and leutinizing hormone begin to rise virtually simultaneously during a preovulatory surge (Lague et al., 1975; Etches and Cunningham, 1976), Bonney and Cunningham (1977) have suggested that the preovulatory surge of leutinizing hormone is initiated by progesterone. Lague et al. (1975), sampling at 20 min intervals, indicated that the peak of estradiol coincides with peak concentrations of progesterone. Johnson and van Tienhoven(1980) reported that peak plasma concentrations of progesterone were consistently found 6 to 2

hr prior to ovulation when laying hens were serially sampled at 2 hr intervals for a period of 34 to 72 hr. A distinct peak of progesterone concentration occurs at 4-6 hr prior to ovulation according to Kappauf and van Tienhoven(1972) and Peterson and Common (1971). For 9 ovulatory cycles the average level was 2.45 ng/ml with a range of 0.5 to 8.8 ng/ml, 2-6 hr before ovulation according to Haynes et al. (1973), and 4-7 hr prior to ovulation according to Cunningham and Furr (1972) and Furr et al. (1973). Lague et al. (1975) reported progesterone peaks preceded ovulation by 4-7 hr. They also found that the progesterone concentrations varied in a pulsatile fashion when blood samples were taken at 20 min intervals at about the time of the expected progesterone peak. Shodono et al. (1975) found the progesterone peak 4-6 hr before ovulation, when the blood samples were taken from hens with regular ovulatory cycle at intervals of 1, 2 or 4 hr throughout 36 hr of the cycle. The values varied from 1 ng/ml to 6 ng/ml.

Kamar et al. (1982) found progesterone peaks 2-3 and 30 hr before oviposition (2.76 $\pm$ 0.68 to 4.51 $\pm$ 1.31 ng/ml). The progesterone surge does not occur on the days when there is no ovulation (Peterson and Common, 1971; Kappauf and Van Tienhoven, 1972; Furr et al., 1973), nor is there any significant daily variation in plasma concentration of progesterone in prepubertal pullets or molting hens (unpublished data, A.L. Johnson). The uptake of progesterone by the pituitary gland, median eminence, and magnum (Kawashima et al., 1978), and the shell gland (Tanaka et al., 1976) is greatest approximately 2 hr before the preovulatory surge of progesterone or 8 hr before the next expected

ovulation. Johnson and van Tienhoven(1981), working on the pharmacokinetics of progesterone in laying and non-laying hens concluded that the preovulatory surge of progesterone is the result of an increase in the production rate and not of a decrease in the metabolic clearance rate of progesterone. They further noticed that the lower metabolic clearance rate of progesterone in laying hens compared with pullets and molting hens is reflected by a smaller volume of distribution in the peripheral compartment, likely to be due to the presence of progesterone receptors of higher affinity and/or binding capacity in laying hens and to differences in extraction efficiency of progesterone - metabolizing organ systems and the shorter half-life of progesterone in avian than in mammalian species.

Leszczynski et al. (1983), while studying sexually mature White Leghorn hens carrying the sex linked restricted ovulator mutation found an unusual low basal level of progesterone, which was associated with failure to lay. Johnson and van Tienhoven(1981) also found low levels of circulating progesterone in immature and molting chicken hens. In Mallard ducks cessation of egg laying induced by stress was associated with low circulating levels of both progesterone and estradiol. Turkeys, non-laying hens, and hens with low egg production also have low levels of circulating progesterone (Mashaly and Wentworth, 1974). Leszczynski et al. (1985) proposed that basal circulating estradiol/progesterone is the best estimator of egg production. Plasma progesterone concentrations have been correlated with eggshell quality at blood sampling times of 20 and 24 hr after oviposition (Hebert et al., 1980). Plasma

concentrations of both progesterone and estradiol are maximal about 3 to 6 hr before ovulation (Shahabi et al., 1975; Lague et al., 1975; Hammond et al., 1980; Johnson and Van Tienhoven, 1980). It might be possible that eggshell quality and estradiol and progesterone concentrations in plasma may be related during this period when hormone concentrations were maximal. Curl et al. (1985) measured the plasma concentrations of progesterone and estradiol-17β at 18, 21 or 24 hr post oviposition between 2 groups of laying hens with high and low shell quality during the 5th, 7th and 9th months of egg production. Egg production and egg weight were not significantly different between shell quality groups. Plasma progesterone and estradiol were not different between shell quality groups or among periods of production, though progesterone and estradiol concentrations were greater at 21 hr post oviposition than at 18 or 24 hr. They concluded no significant association between the plasma concentrations of these hormones and eggshell quality. Williams (1990) studied 2 strains of laying hens; ISA Browns (IB) and a large body strain (Z55). Weekly blood samples were taken from both the strains from 20 to 63 wk of age. Eggshell weight increased with age, and eggshell resistance tended to decline similarly in the last two 28-day laying periods in both strains. Plasma progesterone levels increased from 0.25 ng/ml at 20 wk to 0.55 during the 10th laying period in IB fowl, but was similar at these 2 ages (0.60 and 0.61 ng/ml) in Z55 fowl. In IB fowl, the 10 best laying birds were associated with the higher plasma leutinizing hormone and progesterone concentrations, in Z55 fowl, the

10 worst laying birds were associated with the lower plasma leutinizing hormone levels.

Novero et al. (1989) showed that the preovulatory surge of progesterone is significantly reduced within the first ovulatory cycle 1 day after onset of heat stress on hens, a probable cause in reduced reproductive performance in the hen by disrupting egg production. Novero et al. (1991) further noticed that both progesterone and leutinizing hormone were reduced in the first cycle following the onset of thermal stress suggesting a depressed ovarian function by abolished progesterone secretion.

MATERIALS AND METHODS

The experiment was conducted at the Poultry Science Research and Teaching Center, Michigan State University. Production data were collected from September 1991 to May 1992, while blood collections were carried out till October 1992. Day old birds were procured from Hy-Line Breeders[1]. They were reared according to commercial practices and transferred to the laying house at 16 weeks of age. Fourteen hundred and forty pullets were divided into 3 treatment groups of 480. Each treatment group was housed in a Choretime stair-step, 4 tier cage system. There were 8 lines (4 per side) in each cage system with 12 cages in each line and each cage housed 5 birds for a total of 60 birds per line. Each line served as a replicate within a treatment (8 replicates per treatment group). 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

[1] Hy-Line Indian River Company, 2929 Westown Parkway, West Des Moines, Iowa 50265

treatment 2 evening feed). The calcium source for treatment 3 was 1/2 limestone and 1/2 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 3 treatment groups were fed a commercial layer ration from 18-28 weeks. The experimental diets were fed for about 36 weeks. Feed and water were provided ad libitum throughout the egg production period. The incandescent light was provided 16 hr per day from 8:00 am to midnight. The bulbs were turned off by time clock at midnight until 8:00 am.

EGG PRODUCTION:- Eggs were collected every morning at 11:00 am. Egg production along with mortality for each line was recorded every day. Hen day egg production was calculated by dividing the number of eggs produced by the number of live birds in each line.

EGG SPECIFIC GRAVITY:- Once the hens achieved their peak production, the data on the egg specific gravity was collected on a weekly basis from September 5, 1991 to May 6, 1992. A flat of 30 eggs was randomly collected from each line (replicate) every Wednesday for egg specific gravity

Table 1. Experimental Rations

Ingredients	Ration1[a]	Ration2[b]	Ration3[c]
	(%)	(%)	(%)
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	2666.0000	2676.0000	2676.0000
Crude protein %	16.7703	16.7967	16.7967
Crude fiber %	3.7597	3.7663	3.7663
Calcium %	3.7507	3.7537	3.7537
Total phosphorus %	0.6556	0.5255	0.5255
Available phosphorus %	0.4515	0.3209	0.3254

[a]Ration 1 control , normal calcium and phosphorus
[b]Ration 2 normal calcium (all limestone) , low phosphorus
[c]Ration 3 normal calcium (1/2 limestone & 1/2 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 %
Ration 1 (Control)(normal P with limestone (LS) as Ca)							
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 1/2 LS & 1/2 OS as Ca)[1]							
Limestone	4.50				1.7100		
Oyster shell (OS)	4.50				1.7100		0.0045
TOTAL					3.4200		0.3254

[1] The analysis for ration 3 is identical to ration 2, except for available phosphorus and the 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

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

Nutrients %	Ration 1[1] batch 1	batch 2	Ration 2[2] batch 1	batch 2	Ration 3[3] 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

[1] Ration 1 control, normal calcium and phosphorus
[2] Ration 2 normal calcium (all limestone), low phosphorus
[3] Ration 3 normal calcium (1/2 limestone & 1/2 oyster shell), low phosphorus

Analysis by Michigan Department of Agriculture State Lab.

determination. A total of 720 eggs from the 3 treatments [8 replicates (lines)/treat.] and the specific gravity solutions were stored at 5^{o} C overnight in the poultry farm egg cooler to allow solution and egg temperatures to equalize.

The specific gravity solutions used in this research were 1.065, 1.070, 1.075, 1.080, 1.085 and 1.090. Feed grade salt and tap water were mixed and adjusted to the proper specific gravity using a hydrometer with a range of 1.060 to 1.10 and gradations of 0.0005. The solutions were stored in 5-gallon plastic tubs with sealable lids. These solutions were adjusted if necessary every week just prior to use. Thirty eggs, the sample from 1 line, were placed in a wire mesh basket constructed to fit inside the plastic tubs. The eggs and basket were immersed in each solution starting with the lowest solution of 1.065. Eggs that floated were removed and placed in a flat in front of that solution. The remaining eggs were then immersed in the next highest solution and the procedure repeated. The number of eggs that floated in each solution was recorded. The eggs were assigned the specific gravity of the solution in which they floated, and the eggs that did not float in any solution were assigned a specific gravity of > 1.090. The data thus obtained was processed either as average specific gravity or percent of eggs above 1.080 for statistical analysis.

EGGSHELL THICKNESS:- The eggshell thickness was measured on the last week of every month for the entire period of the experiment . A total of 720 eggs which had previously been used for the specific gravity measurements were broken and a shell chip from the equator of the egg without the shell

membranes was removed and measured for thickness using a micrometer. The average shell thickness was then calculated for each line for each month.

EGG WEIGHT:- The eggs that were collected for the first specific gravity determination were weighed at the beginning of the trial and the eggs collected for the last specific gravity determination were weighed at the end of the trial. A total of 720 eggs were used on each weighing.

BLOOD COLLECTION:- An additional 24 birds representing each treatment were individually caged for blood sampling. All other management practices were the same as those used for the main experiment. They were fed the same experimental diets. 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. 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 hr 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 min. 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 diluent 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.[1])

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 min 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

[1]Sigma Chemical Company, PO Box 14508, St Louis, MO 63178

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 min 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 (Figure 1).

PLASMA ESTRADIOL-17β: Plasma estradiol-17β was determined using an ICN Biomedicals[1], Inc. kit employing ^{125}I radio immunoassay techniques.

ASSAY: This was a 90 min 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[2]) calibrated for ^{125}I . All tubes were counted for 1 min. The results were calculated as follows.

Average counts were calculated for all duplicates. Average non-specific

[1]ICN Biochemicals, Inc. 3300 Hyland Ave, Costa Mesa, CA 92626

[2]TM Analytic, Elk Grove Village, IL

binding counts were subtracted from the averages calculated above to give corrected counts. These corrected counts were divided by the corrected B_O 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 min 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_O = Total binding tube or 0 pg/ml tube of standard curve.
=CPM (0 pg/ml) - CPM (NSB)

Example:

$$\text{10 mg calculation} = \frac{17350-1000}{8298-1000} \times 100$$

% 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 (Figure 2). Using this standard curve, estradiol concentrations at each hour post oviposition (4 samples/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 radio immunoassay 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 min, respectively. The standard curve is presented in Figure 3.

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:- Linear regression analysis was used on the egg specific gravity to find the significance of treatments over the time. The results thus obtained (slopes), were further analyzed by split block design using the mechanics of 3 way analysis of variance with time, treatment and replicates as the 3 main factors. The different means were tested by multiple range test.

Hen day egg production, eggshell thickness and egg weights were analyzed by split block design, using the mechanics of 3 way analysis of variance with time, treatment and replicates as the 3 main factors. The different means were further tested by multiple range test.

Plasma calcium, phosphorus, estradiol and progesterone were analyzed by factorial analysis of variance with time and treatment as the main factors. The different means were analyzed by multiple range test.

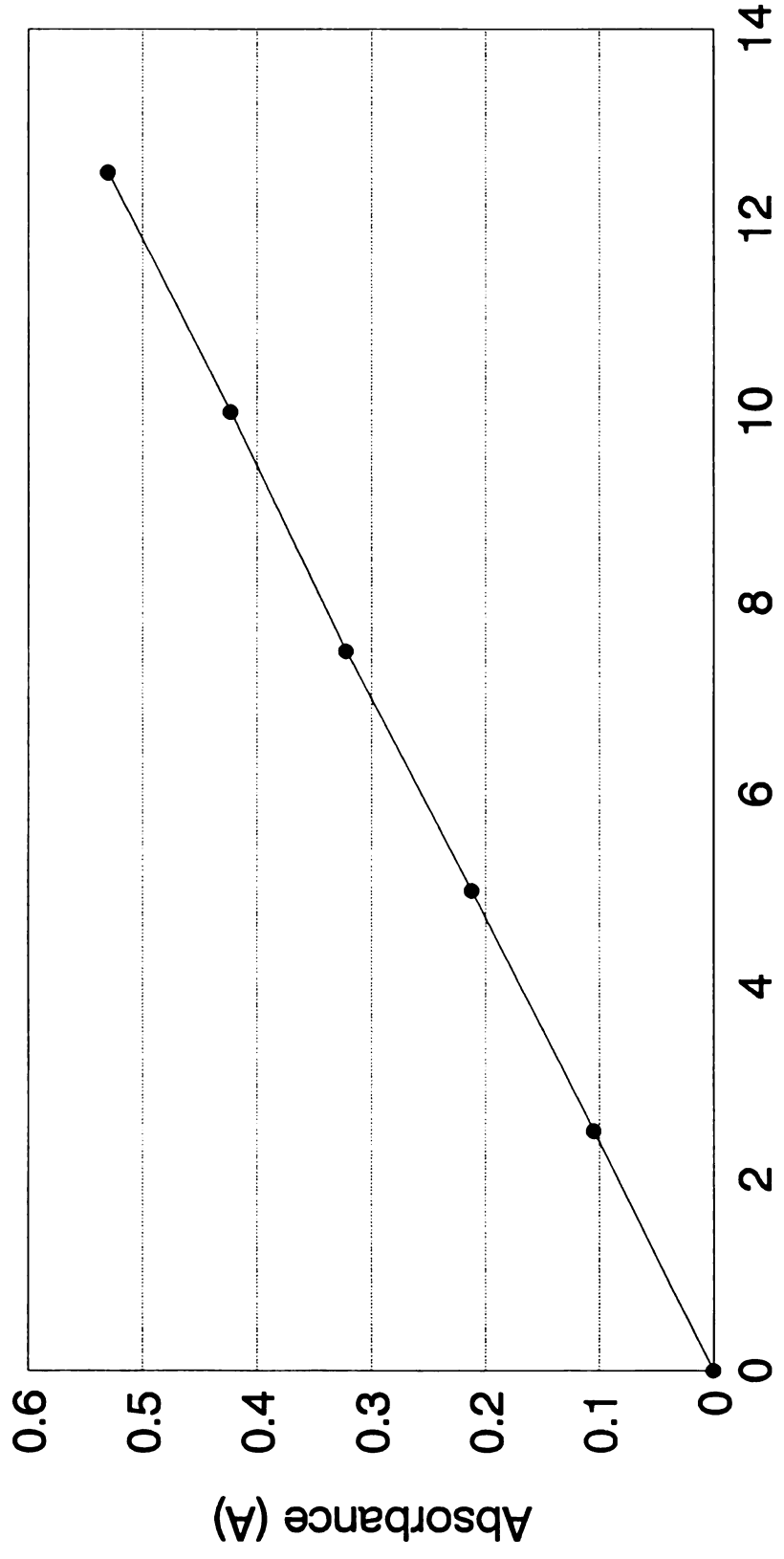

Figure 1. Standard curve for inorganic phosphorus used to measure plasma phosphorus in laying hens.

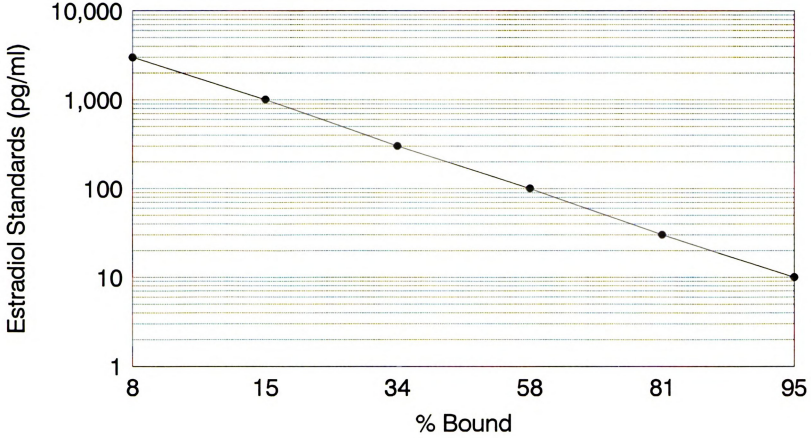

Figure 2. Standard curve for estradiol used to measure plasma estradiol in laying hens.

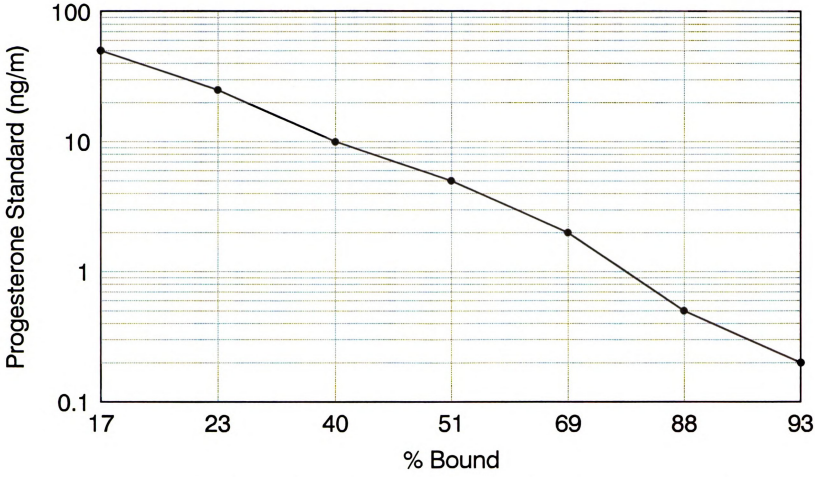

Figure 3. Standard curve for progesterone used to measure plasma progesterone in laying hens.

RESULTS AND DISCUSSION

SPECIFIC GRAVITY

Egg specific gravity measurements were handled in 2 different ways. In the first procedure, based upon the standard commercial practices and literature reviews (Park, 1986), an arbitrary line was drawn among the salt concentrations. All the eggs which had a specific gravity above 1.080 were regarded as "good", while those below 1.085 were considered poor. The percentage of "good" eggs from each replicate was determined. This percentage was then subjected to linear regression analysis to have a comprehensive picture of the correlation between specific gravity and age (weeks of production). The "slopes" (Appendix 1) thus obtained between these 2 traits were further analyzed by analysis of variance (Appendix 2). The treatment means were 0.628, 0.607 and 0.705, respectively, for treatments 1, 2, and 3. There was a significant difference ($P<0.05$) among the treatments. Treatment 3 was better than treatment 1 ($P<0.05$) and treatment 2 ($P<0.01$). Treatment 2 was not different from 1 (Table 4, Figure 4).

Treatment 3, which was composed of control diet in the morning and reduced phosphorus in the evening with 1/2 limestone and 1/2 oyster shell as the calcium source, proved better for specific gravity than treatment 1 which was

a control diet, morning and evening. Treatment 3 was also marginally better than treatment 2, which was composed of the control diet in the morning and reduced phosphorus with all limestone, (as calcium source) in the evening. The improvement in specific gravity by treatment 3 appeared to be a function of the calcium source (oyster shell), rather than reduced phosphorus. This was further evidenced by lack of any difference between treatments 2 and 1, where reduced phosphorus was the only difference between the 2 treatments. These results are in agreement with Moran et al. (1970); Scott et al. (1971); Brister et al.(1981); Makled and Charles (1987) who reported that partial replacement of limestone with oyster shell in the diet improved different aspects of shell quality, including egg specific gravity. These findings are also supported by Hamilton et al. (1985) who found that supplementation of oyster shell to a 3.2% calcium diet increased the egg specific gravity and Keshavarz and McCormick (1991) who found a positive effect on egg specific gravity when 50% of pulverized limestone was replaced with oyster shell as the calcium source in laying hen diets. These results are also in agreement with Salman et al. (1969); Hunt and Chencey (1970); Owings et al. (1977); Reichmann and Connor (1977); El Boushy (1979); and Mikaelian and Sell (1981) who have reported that reducing dietary phosphorus had no significant effect or had an adverse effect on shell quality. They are further substantiated by Keshavarz and Nakajima (1993) who found no 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. These results are also in agreement with Day et al. (1987) who found significantly

Table 4. Mean percentage of eggs with specific gravity above 1.080 by week for birds fed control (treatment 1), limestone with reduced phosphorus in the evening feed (treatment 2) or 1/2 limestone and 1/2 oyster shell with reduced phosphorus in the evening feed (treatment 3). Each number represents an average with standard error of 8 lines/treatment.

Week	Treatment 1	Treatment 2	Treatment 3
1	56.24±5.92	52.10±5.74	52.50±4.44
2	82.78±2.38	81.66±2.10	91.24±1.78
3	79.58±2.03	85.00±2.67	82.91±1.40
4	93.33±1.78	92.50±2.25	98.75±0.61
5	92.50±2.16	92.08±2.18	93.75±1.60
6	63.75±3.45	85.00±3.83	97.07±0.76
7	62.33±6.90	70.00±5.38	87.08±2.31
8	82.50±2.27	49.50±7.80	86.66±2.36
9	87.91±2.88	82.50±2.80	87.08±2.78
10	95.41±1.40	81.25±1.90	96.66±0.63
11	92.08±2.60	93.33±1.90	97.08±0.97
12	85.00±2.74	89.16±1.86	89.99±1.70
13	76.25±4.12	82.91±2.63	86.30±1.50
14	90.00±0.63	92.10±2.60	95.83±2.10
15	85.41±2.35	84.58±2.18	86.25±3.11
16	92.50±1.86	28.75±1.77	90.41±1.77
17	91.66±2.18	90.83±2.42	91.24±1.88
18	73.75±2.22	64.16±3.97	64.58±4.87
19	74.58±4.54	81.25±2.35	78.33±1.86
20	88.33±1.40	86.66±3.50	88.33±2.22
21	49.13±5.59	51.66±5.23	61.23±4.66
22	48.75±4.62	48.74±5.56	52.91±3.85
23	47.08±5.54	55.42±7.20	63.74±4.51
24	82.91±2.91	82.91±1.83	86.66±3.08
25	92.91±2.03	90.41±2.39	93.74±2.22
26	92.08±2.51	90.83±2.33	89.16±3.07
27	66.66±4.17	68.33±5.03	74.58±2.74
28	75.83±4.43	59.16±4.62	72.08±2.66
29	50.00±4.92	44.58±4.71	49.16±4.61
30	74.16±3.97	71.66±3.33	77.08±2.77
31	86.24±5.15	67.87±4.99	70.00±5.59
32	66.66±2.30	87.10±2.66	88.00±2.40
33	33.32±7.01	31.66±4.53	47.50±5.79
34	66.25±4.53	79.20±3.65	88.74±3.50
35	73.33±5.43	73.75±5.54	79.16±5.76
36	37.49±5.10	36.58±5.85	41.24±3.72

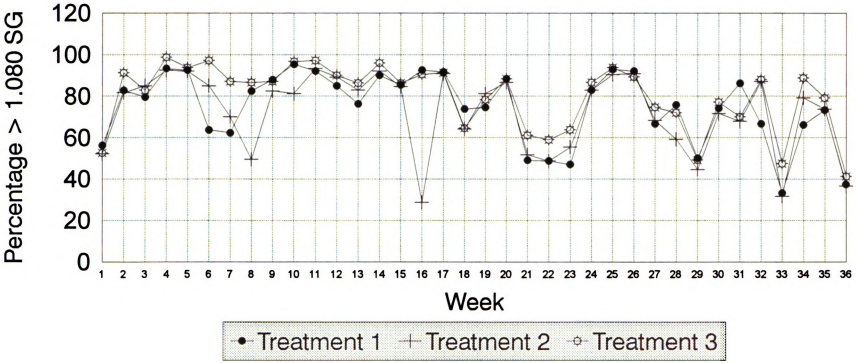

Figure 4. Percentage of eggs with specific gravity above 1.080, by week, for laying hens fed control (treatment 1), limestone with reduced phosphorus in the evening feed (treatment 2) or 1/2 limestone and 1/2 oystershell with reduced phosphorus in the evening feed (treatment 3).

higher egg breaking strength for hens given 0.31% total phosphorus as compared to those given 0.36 to 0.61% total phosphorus. The 2 experimental diets were compared with the control diet (standard layer ration) in an attempt to explore the possible effects of calcium source and reduced phosphorus, particularly in the evening feeds in commercial egg production. The idea behind the reduced phosphorus level in the evening was to decrease the dietary phosphorus availability to the hens at the beginning of the dark period, when egg calcification starts and the hens resort to bone resorption for the extra calcium needed for calcification. With this bone calcium also comes phosphorus as calcium is essentially present in the form of calcium phosphate in medullary bones. This extra phosphorus compensates for reduced dietary phosphorus which prevents phosphorus toxicity and improves eggshell quality. However, this is a complex phenomena and not governed by any single factor. This suggests that mobilization of calcium from bone brought about by reduced phosphorus levels may not explain the occasional slight response in improved eggshell quality reported by some research workers. Roland and Harms (1976), Hamilton and Sibbald (1977), Ousterhout (1980), and Miles et al. (1983) have reported that reducing dietary phosphorus levels below 600 to 700 mg/hen/day produced a small improvement in shell quality (egg specific gravity).

In the second procedure employed to analyze the data on specific gravity, average specific gravity was determined by adding the number of eggs in all the concentrations in each replicate. Similarly, regression analysis was applied on these averages. There was no significant difference among treatments.

Replicates were not different ($P<0.10$) from each other. One possible explanation for this lack of difference among dietary treatments for specific gravity could be the wide variation of observations over the range of salt concentrations. This might have diluted the significant effects of treatments as seen when data was handled according to the first procedure as "above 1.080".

EGG PRODUCTION

A record of daily egg production and mortality was kept for the entire length of the experiment (36 wk). Average weekly hen-day production was then determined by adding the total number of eggs produced by a particular replicate, and dividing by the number of live birds in that replicate, in that particular week. The data thus obtained was analyzed by split block design, using the mechanics of 3 way analysis of variance, with time, treatment and replicates as the 3 main factors (Appendix 3). The treatment means were 0.627, 0.617, and 0.631, respectively, (daily hen-day production) for treatments 1, 2 and 3. There was a significant difference ($P<0.05$) among treatment means, time ($P<0.001$), and replicates ($P<0.05$) for the main effects. The interaction was also significant between time and treatment and replicates ($P<0.01$).

Treatment 3 (reduced phosphorus with 1/2 limestone and 1/2 oyster shell as the calcium source) was significantly better ($P<0.05$) than treatment 2 (reduced phosphorus with limestone as calcium source), but not treatment 1. There was no difference between treatments 1 and 2. Reducing phosphorus alone (T_2 vs. T_1) did not improve egg production, but replacing the limestone with oyster shell

(T_3 vs. T_2) did improve egg production (Table 5, Figure 5). The improved egg production with oyster shell is in agreement with Hamilton et al. (1985) who found increased egg production with oyster shell supplementation. These findings, however, do not agree with Florescu et al. (1986) and Cheng and Coon (1990) who found no difference in egg production with different calcium sources including 4.5% oyster shell meal or limestone replacement with oyster shell, respectively. These results also agree with Antillon (1976) Keshavarz (1986) and Perez and Osa (1987) who found that reducing the available phosphorus in the hen's diet had no effect on egg production. The results, however, disagree with Day et al. (1987) who found higher egg production with 0.31% total phosphorus, compared with 0.36 to 0.61% total phosphorus. The present findings also do not match with those of Vandepopuliere and Lyons (1992) who found depressed egg production when
hens were fed 0.4% total dietary phosphorus compared with 0.5% to 0.7% total phosphorus either from dicalcium phosphate or the regular form of defluorinated phosphate.

EGGSHELL THICKNESS

Eggshell thickness was measured on the last week of every month throughout the entire experiment. All the eggs (a flat of 30 from each replicate, 720 total) were broken uniformly at 1 particular position and the shell membrane was removed before measuring the actual shell thickness. These observations

Table 5. Mean weekly hen day-egg production for laying hens fed control (treatment 1), limestone with reduced phosphorus in the evening feed (treatment 2) or 1/2 limestone and 1/2 oyster shell with reduced phosphorus in the evening feed (treatment 3). Each number represents an average with standard error of 8 lines / treatment.

Weeks	Treatment 1	Treatment 2	Treatment 3
1	0.831±0.011	0.805±0.013	0.833±0.025
2	0.803±0.013	0.776±0.014	0.810±0.033
3	0.700±0.028	0.678±0.021	0.613±0.038
4	0.600±0.048	0.584±0.024	0.670±0.051
5	0.659±0.042	0.654±0.017	0.681±0.042
6	0.693±0.035	0.701±0.015	0.700±0.030
7	0.603±0.034	0.709±0.021	0.705±0.032
8	0.699±0.021	0.621±0.023	0.705±0.026
9	0.763±0.016	0.656±0.029	0.751±0.024
10	0.751±0.016	0.680±0.014	0.726±0.018
11	0.716±0.015	0.735±0.019	0.735±0.018
12	0.653±0.018	0.713±0.013	0.691±0.021
13	0.636±0.022	0.664±0.017	0.659±0.020
14	0.634±0.026	0.640±0.022	0.590±0.031
15	0.563±0.028	0.604±0.030	0.585±0.030
16	0.658±0.00	0.568±0.030	0.590±0.029
17	0.606±0.021	0.585±0.025	0.593±0.023
18	0.594±0.021	0.574±0.028	0.580±0.025
19	0.569±0.014	0.605±0.023	0.578±0.018
20	0.578±0.016	0.588±0.024	0.574±0.015
21	0.576±0.017	0.573±0.029	0.574±0.022
22	0.585±0.019	0.580±0.035	0.580±0.021
23	0.603±0.016	0.585±0.021	0.550±0.023
24	0.581±0.021	0.551±0.033	0.544±0.027
25	0.583±0.031	0.524±0.041	0.561±0.029
26	0.604±0.023	0.604±0.031	0.553±0.019
27	0.561±0.020	0.589±0.030	0.575±0.025
28	0.569±0.023	0.580±0.024	0.561±0.022
29	0.550±0.023	0.560±0.025	0.550±0.016
30	0.555±0.023	0.550±0.031	0.539±0.025
31	0.535±0.021	0.536±0.028	0.554±0.029
32	0.554±0.030	0.560±0.020	0.578±0.030
33	0.565±0.029	0.566±0.018	0.591±0.021
34	0.590±0.023	0.596±0.015	0.631±0.023
35	0.613±0.017	0.623±0.014	0.658±0.015
36	0.641±0.019	0.631±0.012	0.663±0.021

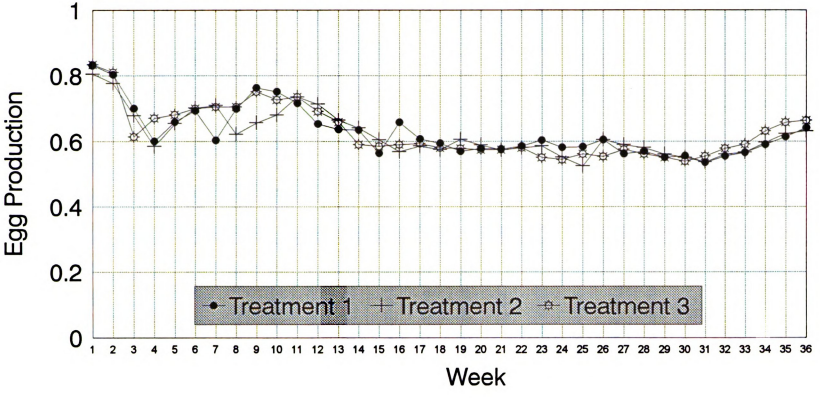

Figure 5. Mean hen-day egg production for laying hens fed control (treatment 1), limestone with reduced phosphorus in the evening feed (treatment 2) or 1/2 limestone and 1/2 oystershell with reduced phosphorus in the evening feed (treatment 3).

were then averaged to get one figure for each replicate in a particular month. The average data thus obtained was analyzed, using a split block design by the mechanics of 3 way analysis of variance, with time, treatment and replicates as the main factors (Appendix 4). The treatment means (overall average of 8 replicates/treatment/9 months) were 0.385, 0.384, and 0.388 mm, respectively, for treatments 1, 2, and 3. The treatment means were not significantly different from each other; nor were the replicates. The main effect of time was significantly different ($P<0.01$). There was an interaction between time and treatment, while time and replicate and treatment and replicate interactions were not different from each other (Table 6, Figure 6).

Lack of difference in eggshell thickness between different dietary treatments is in agreement with Florescu et al. (1986) who could not find any significant difference in eggshell thickness when hens were fed different calcium sources including 4.5% oyster shell meal. These results also agree with Keshavarz and McCormick (1991) who did not find any improvement in shell strength when 50% of pulverized limestone was replaced with oyster shell as the calcium source in laying hen diets during the summer months. These results are not supported by Daghir et al. (1985) who concluded that available phosphorus levels above 0.35% in laying hen diets depressed shell thickness. However, the present results support Keshavarz and Nakajima (1993) who found no difference in eggshell quality when available phosphorus was reduced from 0.4 to 0.2 % with constant 3.5% calcium in laying hen diets.

Table 6: Mean eggshell thickness (mm) by month for hens fed control (treatment 1), limestone with reduced phosphorus in the evening feed (treatment 2) or 1/2 limestone and 1/2 oyster shell with reduced phosphorus in the evening feed (treatment 3). Each number is the average with standard error of 8 lines, 30 eggs/line.

Month	Treatment 1	Treatment 2	Treatment 3
1	0.374±0.002	0.368±0.005	0.374±0.003
2	0.364±0.004	0.373±0.003	0.384±0.002
3	0.387±0.003	0.389±0.002	0.393±0.001
4	0.395±0.005	0.387±0.002	0.393±0.002
5	0.388±0.003	0.389±0.002	0.387±0.002
6	0.386±0.002	0.385±0.003	0.386±0.001
7	0.390±0.002	0.385±0.002	0.390±0.002
8	0.383±0.001	0.388±0.002	0.393±0.003
9	0.394±0.003	0.392±0.002	0.394±0.002

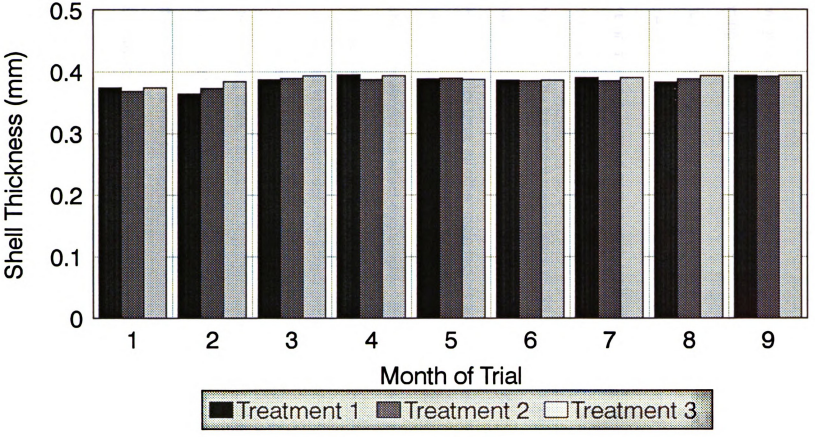

Figure 6. Eggshell thickness (mm) at monthly intervals for laying hens fed control (treatment 1), limestone with reduced phosphorus in the evening feed (treatment 2) or 1/2 limestone and 1/2 oystershell with reduced phosphorus in the evening feed (treatment 3).

EGG WEIGHT

Egg weights were measured only twice throughout the experiment; once in the beginning and again near the end of the experiment; the eggs used for shell thickness were weighed before being broken. Thirty eggs were randomly collected from each replicate, with a total of 240 eggs/treatment or 720 in total. The weights were averaged across each replicate and the data used for 3 way analysis of variance with time, treatments and replicates as the main factors (Appendix 5). The treatment means were 65.35, 65.69, and 65.30 gm, respectively, for treatments 1, 2, and 3. The 2 time means were 62.193 and 68.704. Except for time, all other main factors and interactions were not significantly different from each other. Calcium source and/or reduced phosphorus levels did not affect egg weights. Only the increase in age of the birds increased egg weight. Lack of difference in egg weights by different calcium sources is in agreement with Cheng and Coon (1990), who concluded through a series of experiments that switching from limestone to oyster shell, in short term laying trials, showed no significant differences in eggshell quality or layer performance including egg weight. The increase in egg weight with the age of the hen is a well documented fact. These results are also in agreement with Keshavarz (1986) who found no significant difference in egg weight with other layer performances when hens were fed reducing phosphorus levels from 0.46 to 0.24% with 3.5 to 5.5% calcium. The present findings, however, do not confirm the results of Vandepopuliere and Lyons (1992), who conducted 2

experiments with different phosphorus sources and levels. In experiment 1, hens fed 0.5% total dietary phosphorus produced heavier eggs compared to 0.4% phosphorus. In experiment 2, the same trend was found with depressed egg weight at 0.4% total dietary phosphorus compared with 0.5, 0.6 and 0.7%. This discrepancy in the results could be due to the source or source by level variations in the latter experiments, as defluorinated (coarse or regular) phosphorus and dicalcium phosphate were used as the phosphorus sources.

PLASMA CALCIUM

The concentrations of plasma calcium of the 3 treatments, as determined by atomic absorption spectrophotometry, are presented in Table 7, and Figure 7. The data thus obtained (4 observations/hr) was averaged from each hour in each treatment, and analyzed by factorial analysis of variance with time and treatments as the main factors (Appendix 6). There was a significant difference ($P<0.001$) in time, treatment ($P<0.05$) and time by treatment ($P<0.001$) interactions. Treatment 1 had the highest plasma calcium level at 0 hr among the 3 treatments, reached its peak level (37.075 mg/dl) at 10 hr and then declined towards its lowest level at 22 hr (17.615 mg/dl) post oviposition. Treatment 2 had the lowest plasma calcium level among the 3 treatments at 0 hr (15.585 mg/dl), reached maximum at the 9 hr (31.493 mg/dl) and then dropped to its lowest at 17 hr (15.712 mg /dl) post oviposition. Treatment 3 behaved in a peculiar way. It had its lowest plasma concentration at the 8 hr (14.478 mg/dl), started rising and reached to its peak concentration at the 16 hr (51.847 mg/dl)

Table 7. Mean plasma calcium concentration at hourly intervals post oviposition in laying hens fed control (treatment 1), limestone with reduced phosphorus in the evening feed (treatments 2) or 1/2 limestone and 1/2 oyster shell with reduced phosphorus in the evening feed (treatment 3). Each number represents an average with standard error of 4 observations / hour.

Hour	Treatment 1	Treatment 2	Treatment 3
0	27.13±2.11	15.58±1.95	17.65±1.75
1	26.30±1.57	21.33±1.38	18.80±2.00
2	32.27±3.01	23.77±0.41	25.63±4.33
3	25.59±2.09	18.03±2.21	20.73±2.65
4	27.98±3.55	28.82±3.22	24.16±3.68
5	26.23±2.90	21.07±0.91	19.13±1.91
6	28.43±2.92	21.84±2.14	15.39±2.89
7	29.07±4.31	26.87±2.99	18.86±2.53
8	24.25±3.50	21.38±2.25	14.47±2.65
9	27.44±0.47	31.49±3.18	21.70±5.01
10	37.07±3.06	26.48±3.11	20.98±2.77
11	29.75±4.14	24.99±4.28	24.29±0.07
12	25.81±4.11	18.99±3.27	20.47±2.38
13	24.15±2.48	23.77±2.12	25.74±1.56
14	27.80±2.73	24.39±1.23	28.36±3.13
15	26.65±2.52	25.88±1.83	25.49±2.03
16	25.97±1.40	27.46±6.19	51.84±7.98
17	22.94±2.07	15.71±2.14	22.49±3.20
18	20.15±3.93	22.98±0.82	22.00±4.23
19	23.55±2.50	25.16±3.42	29.13±8.78
20	22.89±1.83	23.49±1.87	24.43±1.10
21	19.14±2.49	23.30±2.49	30.22±6.49
22	17.61±1.03	22.80±2.62	28.89±8.20
23	18.95±1.03	26.98±2.65	21.96±1.61
24	23.28±3.45	24.55±1.64	21.73±1.41

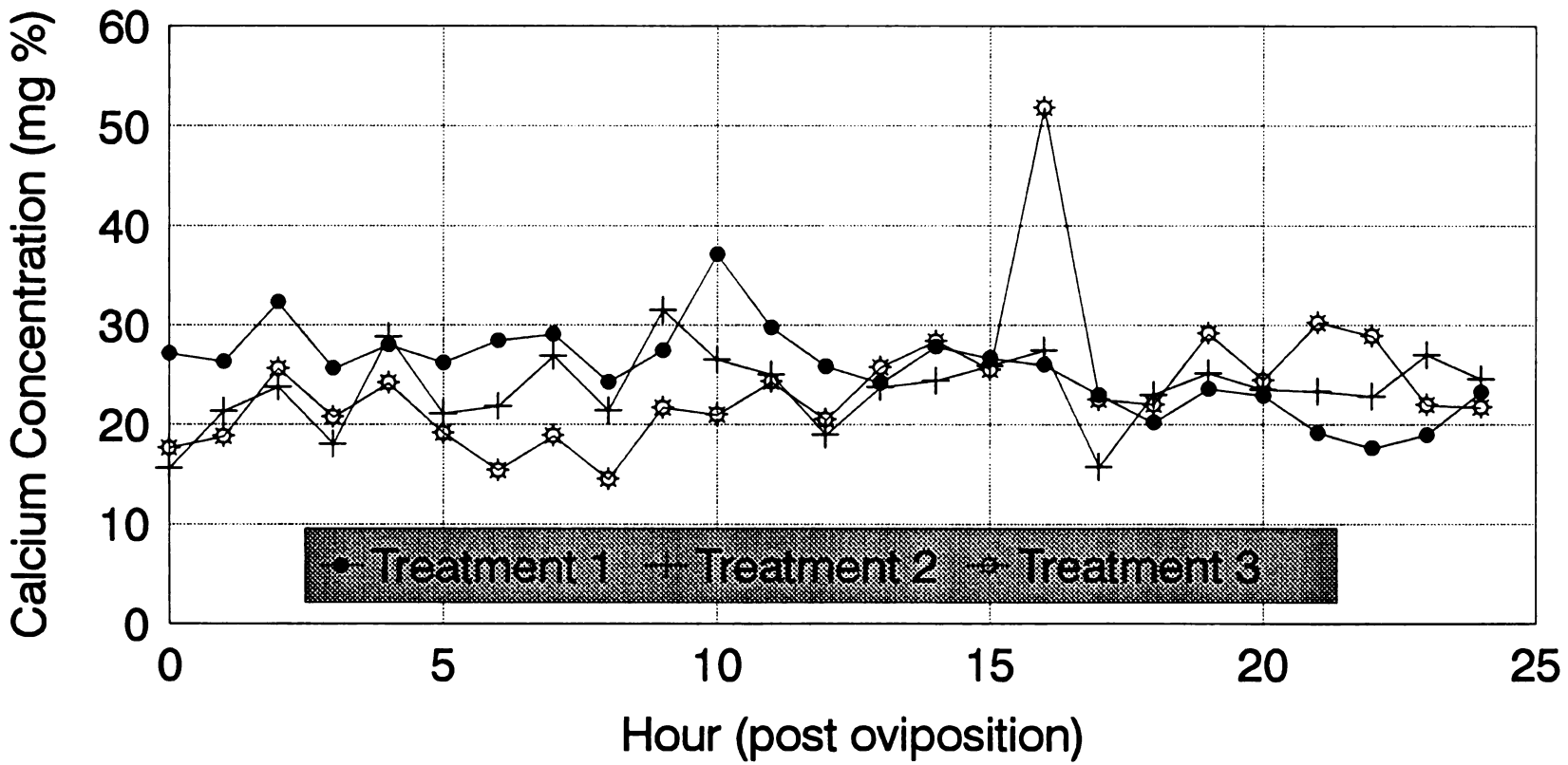

Figure 7. Plasma calcium concentration at hourly intervals post oviposition in laying hens fed control (treatment 1), limestone with reduced phosphorus in the evening feed (treatment 2) or 1/2 limestone and 1/2 oystershell with reduced phosphorus in the evening feed (treatment 3).

post oviposition. This peak concentration, however, was about 2 fold higher than other treatments. Treatment 3 also had the widest plasma calcium range (14.473 to 51.847 mg/dl). 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 2 decades ago. The bird's genetics and 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 1 of their 3 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 treatments 1 and 2 without any significant difference in egg weight or eggshell thickness.

It is hard to explain the effects of treatments in this kind of time course study and experimental design. If examined across each hour, treatment 3 had the lowest plasma calcium, except at the 16th hr as compared to the control diet (treatment 1), but it had the widest variation ranging from approximately 14 to 52 mg/dl. On the average, treatment 3 had 23.786 mg/dl plasma calcium vs. 25.630 mg/dl of treatment 1, while treatment 2 had 23.689 mg/dl plasma calcium across the 24 hr 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 those of Van de Velde et al. (1986), Cheng and Coon (1990), Guinotte and Nys (1991) who found no difference 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 hr post oviposition time, the wide 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 Table 8 and Figure 8. The data was analyzed by factorial analysis of variance with treatment and time as the main factors (Appendix 7). 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 3 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 because all 24 birds in each treatment were bled periodically for each specific hour rather than 1 bird for each different hour post oviposition.

All 3 treatments had 1 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.

Table 8. Mean plasma inorganic phosphorus concentration at hourly intervals, post oviposition in laying hens fed control (treatment 1), limestone with reduced phosphorus in the evening feed (treatment 2) or 1/2 limestone and 1/2 oyster shell with reduced phosphorus in the evening feed (Treatment 3).
Each number represents an average with standard error of 4 observations / hour.

Hour	Treatment 1	Treatment 2	Treatment 3
0	4.98±0.04	5.63±0.41	3.32±0.06
1	5.45±0.18	6.39±0.53	3.48±0.23
2	5.41±0.39	5.63±0.25	3.49±0.26
3	4.03±0.30	5.89±0.79	5.63±1.09
4	5.59±0.46	7.06±0.40	3.17±0.31
5	7.43±0.98	6.01±0.82	2.99±0.45
6	5.84±0.51	4.18±0.58	4.21±0.35
7	6.00±0.99	4.77±0.63	3.73±0.41
8	5.99±0.72	5.43±1.17	4.01±0.62
9	5.08±0.65	8.24±0.68	3.83±0.79
10	7.18±0.86	5.01±0.40	3.69±0.73
11	6.34±0.73	6.49±0.80	3.66±0.43
12	5.54±0.90	4.54±0.26	4.10±0.39
13	5.86±0.32	7.00±0.87	4.56±0.73
14	6.50±0.93	4.98±0.88	5.11±0.69
15	5.61±0.51	5.10±1.01	5.84±0.97
16	6.23±0.36	5.38±0.33	4.71±0.77
17	4.23±0.38	6.01±0.58	3.65±0.64
18	5.08±0.32	4.36±0.74	3.69±0.88
19	5.34±0.63	3.51±0.50	4.90±0.73
20	4.75±0.27	4.66±0.43	4.55±0.95
21	5.88±0.78	3.62±0.39	4.88±0.46
22	3.80±0.35	3.66±0.27	4.57±0.45
23	4.13±0.53	3.95±0.26	4.93±0.28
24	5.13±0.29	4.46±0.34	4.18±0.45

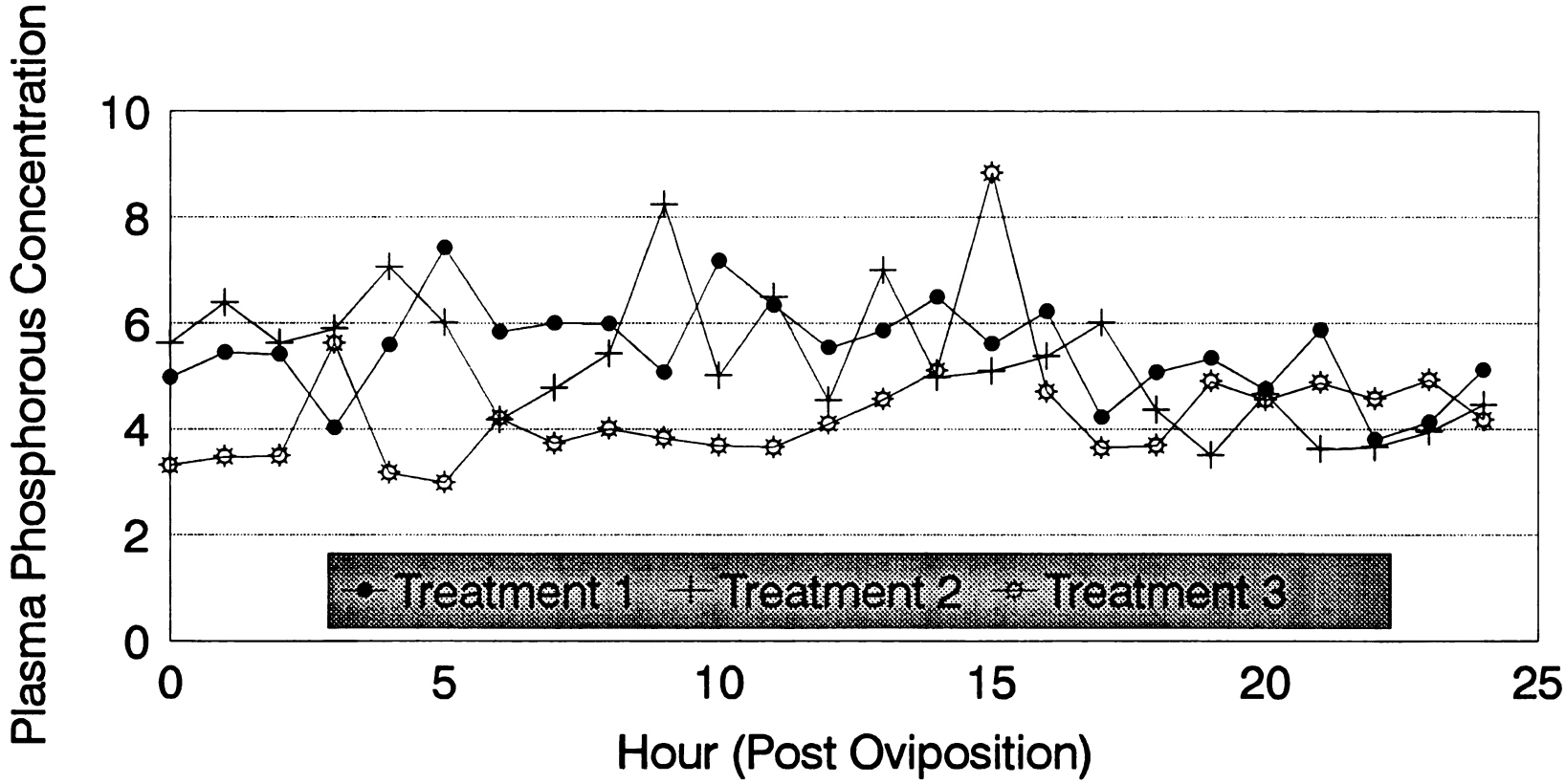

Figure 8. Plasma inorganic phosphorus at hourly intervals post oviposition in laying hens fed control (treatment 1), limestone with reduced phosphorus in the evening feed (treatment 2) or 1/2 limestone and 1/2 oystershell with reduced phosphorus in the evening feed (treatment 3).

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 peak concentration of plasma inorganic phosphorus at 15 hr post oviposition which was 14-14.5 hr after ovulation and reached its lower level at 24 hr post oviposition which was 23-23.5 hr after ovulation. The other 2 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 a 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 those of Keshavarz (1986) who used 3 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 egg shell 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 Miller et al. (1977 a, b) and Mongin and Sauveur (1979b) who confirmed the cyclic nature of the serum phosphorus in relation to the egg laying cycle. They further confirmed the sharp reduction in the serum inorganic phosphorus concentrations 2 to 3 hr prior to oviposition.

PLASMA ESTRADIOL

The average concentration of plasma estradiol at each hour post oviposition is presented in the Table 9 and Figure 9. (Each hour in Figure 9 in all 3 treatments represent an average of 4 samples per hour with duplicate analyses per sample). The data was statistically analyzed by factorial analysis of variance with treatment and time as the main factors (Appendix 8). 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 3 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 3 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 3 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 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 min 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.

Table 9. Mean plasma concentration of estradiol (pg/ml) at each hour post oviposition in laying hens fed control (treatment 1), limestone with reduced phosphorus in the evening feed (treatment 2) or 1/2 limestone and 1/2 oyster shell with reduced phosphorus in the evening feed (treatment 3). Each number represents an average with standard error of 4 observations / hour.

Hour	Treatment 1	Treatment 2	Treatment 3
0	288.25±62.13	173.25±11.79	136.00±19.61
1	185.00±10.21	240.00±18.71	267.50±29.26
2	170.00±12.25	400.00±08.16	263.75±32.10
3	331.25±29.18	421.25±48.49	493.75±85.18
4	325.00±33.23	468.75±82.22	373.75±69.92
5	401.25±85.16	580.00±85.24	368.75±73.33
6	380.00±14.72	577.50±48.33	295.00±20.21
7	457.50±36.14	660.00±90.92	406.25±51.13
8	311.25±32.30	347.50±57.61	375.00±18.37
9	376.25±30.10	490.00±34.16	313.75±72.24
10	415.00±68.62	500.00±40.82	347.50±43.08
11	327.50±57.35	615.00±79.32	370.00±95.66
12	407.50±30.65	392.50±36.83	355.00±35.71
13	261.75±32.78	600.00±63.77	332.50±23.58
14	472.50±46.44	387.50±53.44	330.00±85.54
15	296.25±52.97	505.00±56.20	411.25±59.51
16	365.00±26.30	312.50±106.5	377.50±66.38
17	310.00±40.16	556.25±47.93	453.75±62.36
18	635.00±23.63	492.50±52.50	475.00±79.74
19	477.50±33.26	677.50±149.7	400.00±42.62
20	660.00±20.00	770.00±94.34	642.50±44.04
21	625.00±99.79	630.00±68.07	625.00±61.85
22	520.00±14.14	580.00±57.15	707.25±102.5
23	545.00±22.17	720.00±00.00	585.00±58.52
24	437.50±100.2	596.25±21.54	567.50±65.49

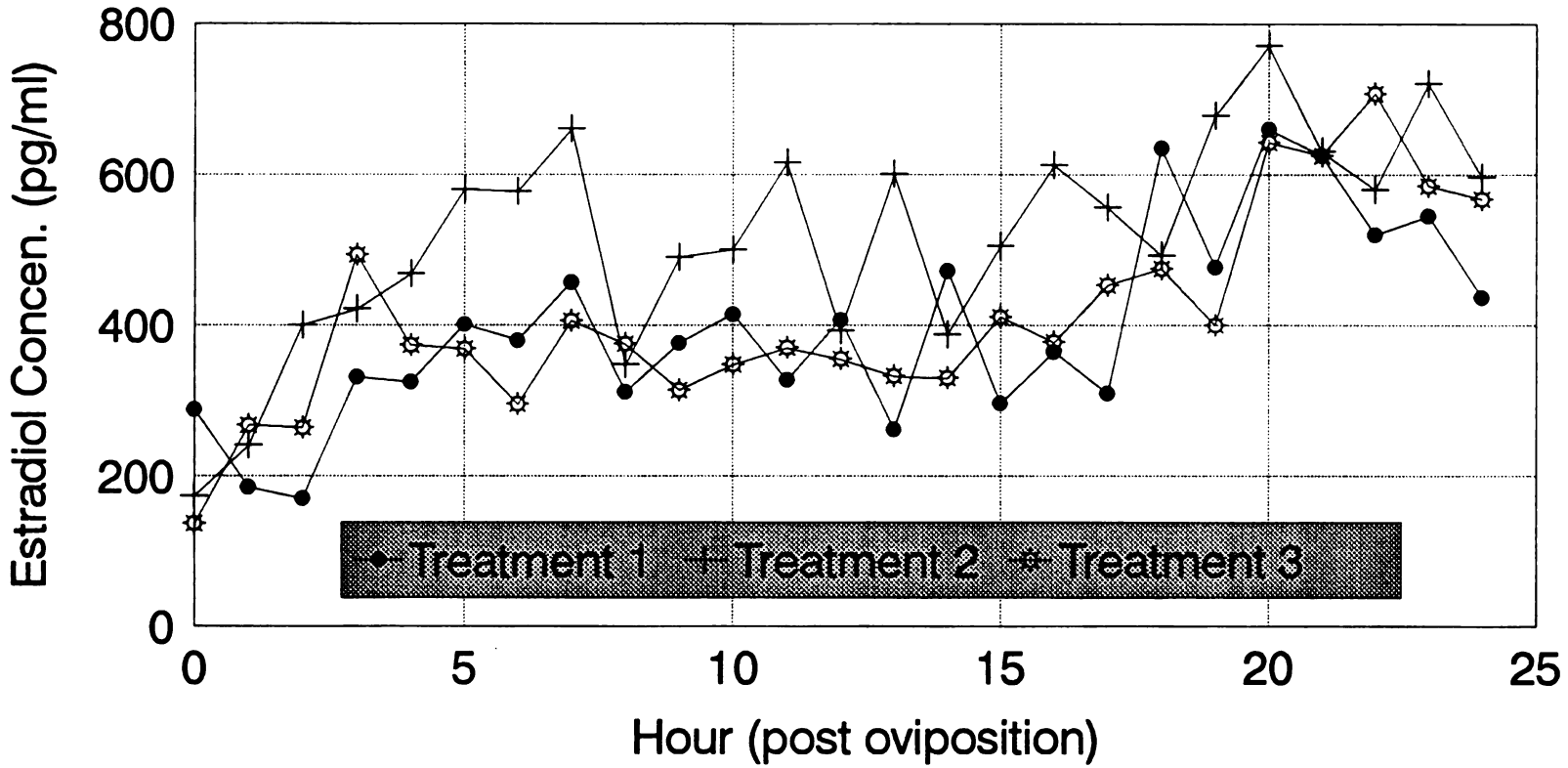

Figure 9. Plasma estradiol at hourly intervals post oviposition in laying hens fed control (treatment 1), limestone with reduced phosphorus in the evening feed (treatment 2) or 1/2 limestone and 1/2 oystershell with reduced phosphorus in the evening feed (treatment 3).

The discrepancies in the results could be due to the methodology adopted by the Shodono group as they took the blood samples within 1 particular clutch and at variable sampling times; while the present study consisted of several clutches and only 1 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 3 treatments. The results are also comparable to those of 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 3 treatments. These values were not different from each other and showed no relationship to eggshell quality. Treatment 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 3 treatments, are presented in Table 10 and Figure 10. The data obtained were statistically analyzed by factorial analysis of variance with treatment and time as the main factors (Appendix 9). 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 3 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 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 3 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) and 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 Shahabi et al. (1975), Lague et al. (1975), Hammond et al. (1980) and 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.

Table 10. Mean concentrations of plasma progesterone (ng/ml) at each hour post oviposition for birds fed control (treatment 1) limestone with reduced phosphorus in the evening feed (treatment 2) or 1/2 limestone and 1/2 oyster shell with reduced phosphorus in the evening feed (treatment 3). Each number represents an average with standard error of 4 observations / hour.

Hour	Treatment 1	Treatment 2	Treatment 3
0	1.44±0.21	0.41±0.05	1.21±0.15
1	0.46±0.09	0.38±0.01	0.56±0.25
2	0.65±0.16	0.44±0.17	0.36±0.01
3	0.17±0.02	1.16±0.16	0.11±0.00
4	0.19±0.00	0.58±0.14	0.28±0.06
5	0.15±0.00	0.49±0.02	0.40±0.09
6	0.24±0.02	0.44±0.11	0.40±0.09
7	0.29±0.04	0.52±0.17	0.44±0.09
8	0.25±0.03	1.16±0.21	0.41±0.08
9	0.23±0.04	0.42±0.00	0.34±0.06
10	0.35±0.04	0.45±0.10	0.30±0.11
11	0.29±0.05	0.52±0.07	0.43±0.38
12	0.23±0.01	1.04±0.19	0.47±0.02
13	0.20±0.01	0.54±0.07	0.42±0.07
14	0.28±0.06	0.66±0.13	0.42±0.03
15	0.10±0.00	0.23±0.03	0.61±0.06
16	0.15±0.00	0.39±0.05	0.43±0.09
17	0.21±0.05	0.85±0.13	0.33±0.03
18	0.58±0.24	0.74±0.13	0.90±0.19
19	1.15±0.26	2.95±0.22	2.68±0.46
20	1.25±0.25	2.78±0.21	2.12±0.51
21	3.94±0.70	2.78±0.21	3.35±0.35
22	3.90±0.36	4.00±0.04	4.61±0.11
23	4.32±0.11	3.15±0.20	2.76±0.45
24	4.50±0.34	2.31±0.18	2.67±0.14

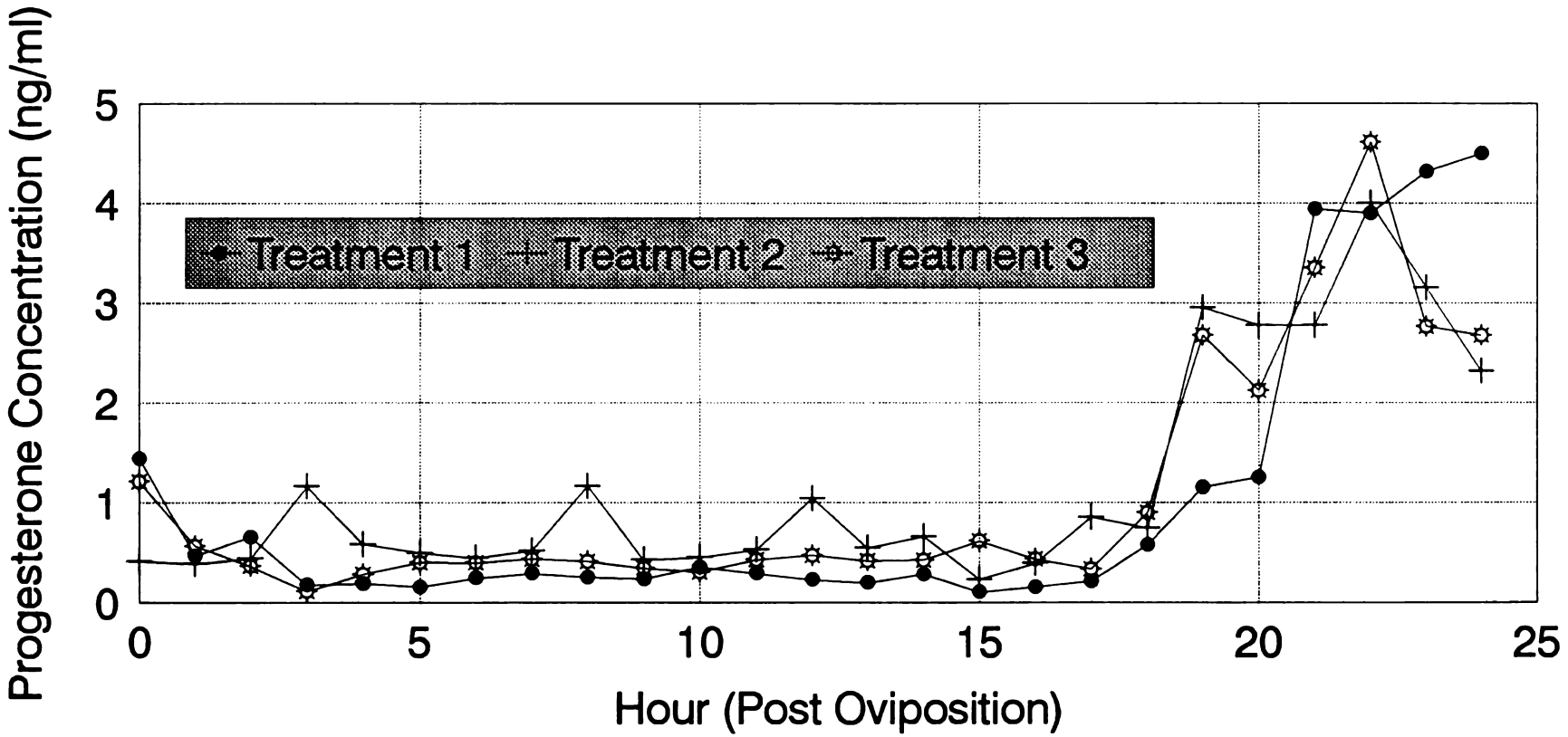

Figure 10. Plasma progesterone concentration at hourly intervals post oviposition in laying hens fed control (treatment 1), limestone with reduced phosphorus in the evening feed (treatment 2) or 1/2 limestone and 1/2 oystershell with reduced phosphorus in the evening feed (treatment 3).

CONCLUSIONS

The results of this study can be summarized as follows:

1. Partial (1/2) replacement of limestone with oyster shell as the calcium source was helpful in improving the eggshell quality as measured by specific gravity, but not eggshell thickness. Oyster shell was also helpful in increasing the hen day egg production. There was no difference in egg weight among the 3 treatments at a given time period. Egg weight did increase as the hen aged.

2. Reducing the available phosphorus level from 0.45% to 0.32% did not show any sign of improvement in eggshell quality (egg specific gravity or shell thickness) or layers' production performance (egg production or egg weight).

3. 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 which had a significantly higher plasma calcium level.

4. 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.

5. Reduced dietary phosphorus and/or calcium source appears to have affected blood levels of estradiol and progesterone, but the results are inconsistent among the 3 treatments. Further study of this may be warranted.

It is concluded that eggshell quality can be improved by partial (1/2) replacement of the usual calcium source, limestone, with oyster shell. Although, reduced phosphorus does not directly improve egg shell 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.

APPENDICES

APPENDICES

Appendix 1. Slopes of Linear Regression Analysis for the % Egg Specific Gravity Above 1.080.

Replicate (lines)	Slopes		
	Treatment 1	Treatment 2	Treatment 3
1	-0.910	-0.486	-0.817
2	-0.506	-0.211	-0.584
3	-0.413	-0.495	-0.730
4	-0.967	-0.865	-1.233
5	-0.293	-0.371	-0.357
6	-0.596	-0.731	-0.579
7	-0.481	-0.780	-0.897
8	-0.856	-0.921	-1.080

APPENDICES

Appendix 2. Analysis of Variance Table for the Slopes of Egg Specific Gravity Above 1.080.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Treatment	2	0.15	0.075	3.65*
Replicate	7	1.17	0.168	8.14**
Error	14	0.29	0.021	
Total	23	1.61		

* $P < 0.05$
** $P < 0.01$

APPENDICES

Appendix 3. Analysis of Variance Table for Hen Day Egg Production

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Factor A[a]	35	4.201	0.120	30.00**
Factor B[b]	2	0.028	0.014	3.50*
AB	70	0.314	0.004	2.00**
Factor C[c]	7	1.608	0.230	77.00**
AC	245	0.774	0.003	1.50**
BC	14	0.336	0.024	12.00**
ABC	490	1.081	0.002	
Total	863	8.341		

* $P < 0.05$
** $P < 0.01$
[a] time
[b] treatment
[c] blocking

APPENDICES

Appendix 4. Analysis of Variance Table for Eggshell Thickness

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Factor A[a]	8	0.011	0.001	8.00**
Factor B[b]	2	0.001	0.0005	4.00*
AB	16	0.002	0.000125	2.78**
Factor C[c]	7	0.001	0.00016	2.64*
AC	56	0.003	0.000053	1.18[NS]
BC	14	0.001	0.000071	1.58[NS]
ABC	112	0.005	0.000045	
Total	215	0.025		

* $P < 0.05$
** $P < 0.01$
[NS] non-significant
[a] time
[b] treatment
[c] blocking

APPENDICES

Appendix 5. Analysis of Variance Table for Egg Weight

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Factor A[a]	1	508.756	508.756	256.42**
Factor B[b]	2	1.475	0.738	0.37[NS]
AB	2	3.969	1.985	2.43[NS]
Factor C[c]	7	23.808	3.401	2.25[NS]
AC	7	10.556	1.508	1.85[NS]
BC	14	4.937	0.353	0.43[NS]
ABC	14	11.424	0.816	
Total	47	564.926		

** $P < 0.01$
[NS] non significant
[a] treatment
[b] time
[c] blocking

APPENDICES

Appendix 6. Analysis of Variance Table for Plasma Calcium

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Factor A[a]	24	3169.161	132.048	3.1552***
Factor B[b]	2	226.448	133.224	3.1833*
AB	48	4871.640	101.493	2.4251***
Error	225	9416.488	41.851	
Total	299	17723.737		

* P< 0.05
*** P < 0.001
[a] hour
[b] treatment

APPENDICES

Appendix 7. Analysis of Variance Table for Plasma Inorganic Phosphorus

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Factor A[a]	24	69.947	2.91	1.9095**
Factor B[b]	2	100.343	50.172	32.8712***
AB	48	197.103	4.10	2.6903***
Error	225	343.420	1.526	
Total	299			

** $P < 0.01$
*** $P < 0.001$
[a] hour
[b] treatment

APPENDICES

Appendix 8. Analysis of Variance Table for Plasma Estradiol

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Factor A[a]	24	4020446	167519	12.2815***
Factor B[b]	2	882976	441488	32.3672***
AB	48	1239866	25831	1.8937*
Error	225	3068993	13640	
Total	299	9212281		

* $P < 0.05$
*** $P < 0.001$
[a] hour
[b] treatment

APPENDICES

Appendix 9. Analysis of Variance Table for Plasma Progesterone

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value
Factor A[a]	24	69.947	2.914	1.9095**
Factor B[b]	2	100.343	50.172	32.8712***
AB	48	197.103	4.106	2.6903***
Error	225	343.420	1.526	
Total	299			

** $P < 0.01$
*** $P < 0.001$
[a] hour
[b] treatment

BIBLIOGRAPHY

Abe, E., R. Tanabe, T. Suda, S. Yoshiki, H. Horikawa, T. Masmura, and M. Sughara. 1979. Circadian rhythm of 1,25-dihydroxy vitamin D_3 production in egg-laying hens. Biophys. Biochem. Res, Commun. 88:500-507.

American Association of Avian Pathologists, 1987. 1986 summary of commercial poultry disease reports. Avian Dis. 31:926-982.

Antillon, A. 1976. Ph. D Thesis. Pathology of caged laying hens fed different levels of calcium, phosphorus, and vitamin D3. Cornell University, New York. Pages 173.

Antillon, A., M. L. Scott, L. Krook, and R. H. Wasserman, 1977. Metabolic response of laying hens to different dietary levels of calcium, phosphorus and vitamin D_3. Cornell Vet. 67:413-414.

Arscott, G.H., P. Rachapaetayakom, P.E. Benier, and F.W. Adams. 1962. Influence of ascorbic acid, calcium, and phosphorus on specific gravity of eggs. Poultry Sci. 41:485-488.

Bell, D. J., and W. G. Siller, 1962. Cage layer fatigue in brown leghorns. Res. Vet. Sci. 3:219-229.

Benoit, J. and J. Calvert. 1945. Role indispensable des parathyroids dans la calcification de l'osfolliculinque chez le canard domestique. C.R. Seanc. Soc. Biol. 139:743

Bezpa, J., Dupras, C.A. and Dowling, J.J. 1974. Supplemental feeding of Oyster shell to improve eggshell quality. Poultry Sci. 53:1901 (Abstr.).

Bletner, J.K. and McChee, G.C. 1975. Effect of phosphorus on egg specific gravity and other production parameters. Poultry Sci. 54:1736 (Abstr.).

Boelkins, J.N., Muller, W.J. and Hall, K.L. 1973. Cardiac output distribution in the laying hen during shell formation. Comp. Biochem. and Phys. 46 A: 735-743.

Bolden, S.L. and L.S. Jensen. 1985. Effect of dietary calcium level and ingredient composition on plasma calcium and shell quality in laying hens. Poultry Sci. 64:1449-1505.

Bonney, R.C. and F.J. Cunningham. 1977. The sensitizing effect of oestradiol on the response of the anterior pituitary gland of the domestic fowl to luteinizing hormone. J. Endocrinol. 72:16P (Abstr.)

Brister, R.D. Jr., S.S. Linton, and C.R. Creger. 1981. Effects of dietary calcium sources and particle sizes on laying hens performance. Poultry Sci. 60:2643-2654.

Candlish, J. R., 1981. The formation of mineral and organic matrix of fowl cortical and medullary bone during shell calcification. Br. Poult. Sci. 12:119-127.

Castillo, L., Y. Tanaka, H.F. DeLuca, and M.L. Sunde. 1977. The stimulation of 25-hydroxyvitamin D_3-1α-hydroxylase by estrogen. Arch. Biochem. Biophys. 60:311-317.

Charles, O.W., S.E. Duke, and S. Savage. 1984. The effect of calcium solubility on the productive response of leghorn hens. in: Proceedings XVII World's Poultry Congress, World's Poultry Science Association, Helsinki, Finland. Pages 399-401.

Cheng, T.K. and C.N. Coon. 1990. Effect on layer performance and shell quality of switching limestones with different solubilities. Poultry Sci. 69:2199-2203.

Clagett, C.O., E.G. Buss, and Y. Tamaki. 1977. Egg shell quality: calcium metabolism in thick and thin shell genotypes. Poultry Sci. 56:1703 (Abstr.)

Common, R.H. 1932. Mineral balance studies on poultry. J. Agric. Sci. 23:576-594.

Common, R.H. 1933. Observations in the mineral metabolism of pullets. J. Agric. Sci. 23:555-750.

Common, R.H. 1936. Serum phosphatase in the domestic fowl. J. Agric. Sci. 26:492-508.

Couch, J. R., 1955. Cage layer fatigue. Feed Age. 5:55-57.

Cunningham, F.J. and B.J.A. Furr. 1972. Plasma levels of luteinizing hormone and progesterone during the ovulatory cycle of the hen. In: "Egg Formation and Production" (B.M. Freeman and P.E. Lake, eds.). pp.51-64. British Poultry Science Ltd., Edinburgh.

Curl, J.S., R. Thayer, R.P. Wettemann, and R. Morrison. 1985. Preovulatory concentrations of progesterone and estradiol in plasma and their relationships with eggshell quality in the laying hen. Poultry Sci. 64:2383-2387.

Daghir, N.J., M.T. Farran, and S.A. Kaysi. 1985. Phosphorus requirements of laying hens in a semiarid continental climate. Poultry Sci. 64:1382-1384.

Damron, B.L., A.R. Eldred, and R.H. Harms. 1974. The relationship of dietary phosphorus to eggshell quality. Poultry Sci. 53:1916 (Abstr.).

Day, E. J., B. C. Dilworth, and S. Omar. 1987. Effect of varying levels of phosphorus and live yeast culture in caged layer diets. Poultry. Sci. 66:1402-1410.

Douglas, C.R., R.H. Harms, M.D. Carpenter, and T.C. Chaillo. 1978. Performance of Leghorn-type hens fed a broiler-type diet with or without estrogenic hormone during the growth period. Poultry Sci. 57:1135 (Abstr.).

Draper, H. H., Ten-Lin Sie, and J. G. Bergan. 1972. Osteoperosis in aging rats induced by high phosphorus diets. J. Nutr. 102:1133-1142.

El Boushy, A.R. 1979. Available phosphorus in poultry. 1. Effect of phosphorus levels on the performance of laying hens and their egg quality, hatchability, bone analysis and strength in relation to calcium and phosphorus in blood plasma. Neth. J. Agric. Sci. 27:176-182.

Edwards, Jr. H.M. 1978. Phosphorus requirements of laying hens. Proceedings Georgia Nutrition Conference. pp.67-73.

Etches, R.J. and F.J. Cunningham. 1976. The interrelationship between progesterone and luteinizing hormone during the ovulation cycle of the hen (Gallus domesticus). J. Endocrinol. 71:51-58.

Etches, R. J., 1987. Calcium logistics in the laying hens. J. Nutr. 117:619-628.

Farmer, M., D.A. Roland, Sr. and A.J. Clark. 1986. Influence of dietary calcium on bone calcium utilization. Poultry Sci. 65:337-344.

Feinberg, J.C., J.S. Hughes, and H.M. Scott. 1937. Fluctuations of calcium and inorganic phosphorus in the blood of the laying hen during the cycle of one egg. Poultry Sci. 16:132-134.

Florescu, S., S. Paraschiv, and A. Florescu. 1986. Utilization of calcium from different sources in feeding laying hens. Revista de Crestera Aniimalelor. 36:45-51.

Francis, D. W., 1957. Strain differences in the incidence of cage layer fatigue. Poultry Sci. 36:181-183.

Fraps, R.M. 1955a. Egg production and fertility in poultry. In: Hammond, J., Ed. Progress in the Physiology of Farm Animals, Butterworth Scientific Publications, London. 2:661-740

Fraps, R.M. 1955b. The varying effects of sex hormones in birds. Mem. Soc. Endocr. 4:205-219.

Fraps, R.M. 1961. Ovulation in the domestic fowl. In: Vilee, C.A., Ed. Control of Ovulation. Pergamon Press, Oxford, London, New York. pp.133-162

Frost, T.J. and D.A. Roland, Sr. 1991. The influence of various calcium and phosphorus levels on tibia strength and eggshell quality of pullets during peak production. Poultry Sci. 70:963-969.

Furr, B.J.A., R.C. Bonney, R.J. England, and F.J. Cunningham. 1973. Luteinizing hormone and progesterone in peripheral blood during the ovulatory cycle of the hen (Gallus domesticus). J. Endocrinol. 57: 159-169.

Garlich, J.D. 1979. The phosphorus requirements of laying hens. Proc. Georgia Nutr. Conf. pp.104-114.

Gillis, M.B., L.C. Norris, and G.F. Heuser. 1953. Phosphorus metabolism and requirements of hens. Poultry Sci. 32:977-984.

Grunder, A.A., R.B. Guyer, E.G. Buss, and C.O. Clagett. 1978. Calcium-binding in serum: quantitative differences between thick and thin shell lines. Poultry Sci. 57:1139-1140.

Grunder, A.A., R.B. Guyer, E.G. Buss, and C.O. Clagett. 1980. Effect of estradiol on calcium and calcium-binding in serum of thick and thin-shell lines of chickens. Poultry Sci. 59:2276-2281.

Grunder, A.A., K.G. Hollands, and C.P.W. Tsang. 1983. Plasma estrogen, calcium and shell quality in two strains of White Leghorns. Poultry Sci. 62:1294-1296.

Guenter, W. 1980. Dietary phosphorus and laying hen performance. Poultry Sci. 59:1615 (Abstr.).

Guinotte, F. 1987. Efficacite de differentes sources de calcium pour l' ossification du poulet et la qualite de la coquille de l' out. Role de la secretion acide dans leur utilization digestive. in : Rapport Bibliographique. Station de Recherches Agronomique, Nouzilly Monnaie, France. Pages 1-56.

Guinotte, F. and Y. Nys. 1991. Effects of particle size and origin of calcium sources on eggshell quality and bone mineralization in egg-laying hens. Poultry Sci. 70:583-592.

Guyer, R.B., A.A. Grunder, E.G. Buss, and C.O. Clagett. 1980. Calcium-binding proteins in serum of chickens: vitellogenin and albumin. Poultry Sci. 59:874-879.

Hamilton, R.M.G. and I.R. Sibbald. 1977. The effect of dietary phosphorus on productive performance and egg quality of ten strains of White Leghorns. Poultry Sci. 56:1221-1228.

Hamilton, R.M.G. 1980. The effects of dietary phosphorus, vitamin D_3, and 25-hydroxy vitamin D_3 levels on feed intake, productive performance, and eggshell quality in two strains of force molted White Leghorns. Poultry Sci. 56:1221-1228.

Hamilton, R.M.G., R.W. Fairfull, and R.S. Gowe. 1985. Use of particulate limestone or oyster shell in the dietary regimen of White Leghorn hens. Poultry Sci. 64:1750-1762.

Hammond, R.W., D.M. Olson, R.B. Frenkel, H.V. Biellier, and F. Hertelendy. 1980. Prostaglandins and steroid hormones in plasma and ovarian follicles during the ovulation cycle of the domestic hen (Gallus domesticus). Gen. Comp. Endocrinol. 42:195-202.

Harms, R.H., C.R. Douglas, and P.W. Waldroup. 1961. The effects of feeding various levels and sources of phosphorus to laying hens. Florida Agri. Exper. Stat. Bull.. 644, pp.1-22.

Harms, R.H. and R.D. Miles. 1980. Correlation of specific gravity of eggs and blood phosphorus in laying hens fed different levels of calcium phosphorus and sodium bicarbonate. Poultry Sci. 59:1618-1619.

Harms, R. H. 1982a. The influence of nutrition on eggshell quality. Part II: Phosphorus. Feedstuffs, May 17, pp.25-26.

Harms, R.H. 1982b. The influence of nutrition on eggshell quality. Part III: Electrolyte balance. Feedstuffs, May 17, pp.25-28.

Hartel, H. 1989. Evaluation of the dietary interaction of calcium and phosphorus in the high producing laying hen. Br. Poult. Sci. 31:473-494.

Haynes, N.B., K.J. Cooper, and M.J. Kay. 1973. Plasma progesterone concentrations in the hen in relation to the ovulatory cycle. Br. Poult. Sci. 14:349-357.

Hebert, J.A., R.B. Abdullah, and D.G. Satterlee. 1980. Factors inherent in the laying hen that affect eggshell quality. Poultry Sci. 59:1565. (Abstr.).

Hertelendy, F. and T.G. Taylor. 1961. Changes in blood calcium associated with egg shell calcification in the domestic fowl. 1. Changes in total calcium. Poultry Sci. 40:108-114.

Hodges, R.D. 1970. Blood pH and cation levels in relation to eggshell formation. Annal. Biol. Anim. Bioch. Biophys. 10:199-213.

Holtea, J., S. Lazar, and T. Balan. 1985. Effects of different methods of supplying calcium to laying hens on utilization in egg production. Zootec.- Med. Vet. 29:39-40.

Hopkins, J.R., A.J.Ballantyne, and J.L.O. Jones. 1987. Dietary phosphorus for laying hens. In: Recent Advances in Animal Nutrition. pp.39-46. Butterworth, London.

Hunt, J.R. and H.W.R. Chencey. 1970. Influence of dietary phosphorus on shell quality. Br. Poult. Sci. 11:259-267.

Hurwitz, S. and P. Griminger. 1962. Estimation of calcium and phosphorus requirement in laying hens by balance techniques. J. Sci. Food Agri. 3:185-191.

Hurwitz, S., 1965. Calcium turnover in different bone segments of laying hens. Am. J. Phsiol. 208:203-207.

Hurwitz, S. and A. Bar. 1965. The absorption of calcium and phosphorus along the gastrointestinal tract of the laying fowl as influenced by dietary calcium and eggshell formation. J. Nutr. 86:433-438.

Johnson, A.L. and A. van Tienhoven. 1980. Plasma concentrations of six steroids and LH during the ovulatory cycle of the hen, Gallus domesticus. Biol. Reprod. 23:386-393.

Johnson, A.L. and A. van Tienhoven. 1981. Pharmacokinetics of progesterone in laying and non laying hens (Gallus domesticus). J. Endocr. 89:1-12.

Kamar, G.A.R., H.A.M. Hassan, M.A. Kicka, R.K.R. Stino, and M.M. El Nodi. 1982. Sex hormone during the laying cycle of the hen. Egyp. J. Anim. prod. (2):183-190.

Kaetzel, D.M. and J.S. Soares. 1984. The effect of dietary cholecalciferol steroids and gonadal hormone implantation on plasma 1,25-dihydroxycholecalciferol and bone calcification in preovulatory and senescent female Japanese quail. Nutr. Res. 4:631-632.

Kappauf, B. and A. Van Tienhoven. 1972. Progesterone concentrations in peripheral plasma of laying hens in relation to the time of ovulation. Endocrinology. 90:1350-1355.

Kawashima, M., M. Kamiyoshi, and K. Tanaka. 1978. A Cytoplasmic progesterone receptor in hen pituitary and hypothalamic tissues. Endocrinology. 102:1207-1213.

Kenny, A.D. 1976. Vitamin D metabolism: Physiological regulation in egg-laying Japanese quail. Am. J. Physiol. 230:1609-1615.

Keshavarz, K. 1986. The effects of dietary levels of calcium and phosphorus on performance and retention of these nutrients by laying hens. Poultry Sci. 65:114-121.

Keshavarz, K. and C.C. McCormick. 1991. Effect of sodium aluminosilicate, oyster shell, and their combinations on acid-base balance and eggshell quality. Poultry Sci. 70:313-325.

Keshavarz, K. and S. Nakajima. 1993. Re-evaluation of calcium and phosphorus requirements of laying hens for optimum performance and eggshell quality. Poultry Sci. 72:144-153.

Kuhl, M.J., D.P. Holder, and T.W. Sullivan. 1977. Influence of dietary calcium level, source and particle size on performance of laying chickens. Poultry Sci. 56:605-611.

Lague, P.C., A. Van Tienhoven, and F.J. Cunningham. 1975. Concentrations of estrogens, progesterone and LH during the ovulatory cycle of the laying chicken. Biol. Reprod. 12:590-598.

Lawson, D.E.M., D.R. Fraser, E. Kodicek, H.R. Mossis, and D.H. Williams. 1971. Identification of 1,25-Dihydroxycholecalciferol, a new kidney hormone controlling calcium metabolism. Nature (London) 230, 228-230.

Lennards, R., D.A. Sr. Roland, and J.A. McGuire. 1981. The relationship of serum calcium to shell weight and other criteria in hens laying a low or high incidence of shell-less eggs. Poultry Sci. 60:2501-2505.

Leszczynski, D.E., J. Pikul, and F.A. Kummerow. 1983. Relationship of circulating estrogen and progesterone to plasma lipids and egg productivity in chicken hens. Poultry Sci. 62:1457 (Abstr.).

Leszczynski, D.E., R.C. Hagan, J.J. Bitgood, and F.A. Kummerow. 1985. Relationship of plasma estradiol and progesterone levels to egg productivity in domestic chicken hens. Poultry Sci. 64:545-549.

Lobaugh, B., I.G. Joshua, and B.J. Mueller. 1981. Regulation of calcium appetite in broiler chickens. J. Nutr. 111:298-306.

Makled, M.N. and O.W. Charles. 1987. Eggshell quality as influenced by sodium bicarbonate, calcium source, and photoperiods. Poultry Sci. 66:705-712.

March, B.E. and M. Amin. 1981. Dietary limestone versus extra dietary oyster shell as calcium supplements to different layer diets. Poultry Sci. 60:591-597.

Mashaly, M.M., and B.C. Wentworth. 1974. A Profile of progesterone in turkey sera. Poultry Sci. 53:2030-2035.

McMurtry, P.J. and L.J. Forbish. 1981. Blood calcium and magnesium response to estrogen in caged and floor housed turkey. Poultry Sci. 60:1688 (Abstr.).

Meyer, R., R.C. Baker, and M.L. Scott. 1973. Effects of hen eggshell and other calcium sources upon eggshell strength and ultrastructure. Poultry Sci. 52:949-955.

Mikaelian, K.S. and J.L. Sell. 1981. Performance of laying hens fed various phosphorus levels continuously or phase fed decremental phosphorus levels. Poultry Sci. 60:1916-1924.

Miles, R.D. and R. H. Harms. 1982. Relationships between egg specific gravity and plasma phosphorus from hens fed different dietary calcium, phosphorus, and sodium levels. Poultry Sci. 61:175-177.

Miles, R.D., A.P.T. Costa, and R.H. Harms. 1983. The influence of dietary phosphorus levels on laying hen performance, egg shell quality, and various blood parameters. Poultry Sci. 62:1033-1037.

Miller, E.R, R.H. Harms, and H.R. Wilson. 1977a. Cyclic changes in serum phosphorus of laying hens. Poultry Sci. 56:586-589.

Miller, E.R., R.H. Harms, and H.R. Wilson. 1977b. Serum calcium and phosphorus levels in hens relative to the time of oviposition. Poultry Sci. 56:1501-1503.

Miller, S. C.,1981. Osteoclast cell-surface specialization and nuclear kinetics during egg laying in Japanese quail. Am. J. Anat. 162:35-43.

Mongin, P. and B. Sauveur. 1974. Voluntary food and calcium intake by laying hens. Br. Poult. Sci. 15:349-359.

Mongin, P. and B. Sauveur. 1975. Solidite de la coquille de l'Quef: Alimentation calcique separee. Courr. Avicole. 553:1-9.

Mongin, P. and B. Sauveur. 1979a. The specific appetite of the domestic fowl. in: Food Intake Regulation in Poultry. Boorman, K.N. and B.M. Freeman. eds. Br. Poult. Sci Ltd. Edinburgh, Scotland. Pages 171-189.

Mongin, P. and B. Sauveur. 1979b. Plasma inorganic phosphorus concentration during egg-shell formation. Effect of the physical form of the dietary calcium. Br. Poult. Sci. 20:401-412.

Moran, Jr., E.T., A. Eyal, and J.D. Summers. 1970. Effectiveness of extra dietary Ca supplements in improving eggshell quality and the influence of added phosphorus. Poultry Sci. 49:1011-1022.

Mueller, W.J., R. Schraer, and H. Schraer. 1964. Calcium metabolism and skeletal dynamics of laying pullets. J. Nutr. 84:20-26.

Muir, F.V., R.W. Gerry, and P.C. Harris. 1975. Effects of various sources and sizes of calcium carbonate on egg quality and laying house performance of red x rock sex-linked females. Poultry Sci. 54:1898-1904.

NRC (National Research Council). 1971. Nutrient requirements of poultry. 6th Rev. ed. Natl. Acad. Sci., Washington, D.C. Pages 2-4.

NRC (National Research Council. (1977). Nutrient requirements of poultry. 7th Rev ed. Natl. Acad. Sci., Washington, D.C. Pages 2-10.

Navickis, R.J., B.S. Katzenellenbogen, and A.V. Nalbandov. 1979. Effects of the sex steroid hormones and vitamin D_3 on calcium-binding proteins in the chicken shell gland. Biol. Reprod. 21:1153-1162.

Nott, H., F.R. Morris, and T.E. Taylor. 1967. Utilization of phytate phosphorus by laying hens and young chicks. Poultry Sci. 46:1301(Abstr.).

Novero, R.P., M.M. Beck, E.W. Gleaves, and K.M. Eskridge. 1989. Plasma progesterone during the ovulatory cycle of heat stressed hens. Poultry Sci. 68(Suppl. 1):106 (Abstr.).

Novero, R.P., M.M. Beck, E.W. Gleaves, A.L. Johnson, and J.A. Deshazer. 1991. Plasma progesterone, lutenizing hormone concentrations, and granulosa cell responsiveness in heat-stressed hens. Poultry Sci. 70:2335-2339.

Ousterhout, L.E. 1980. Effect of calcium and phosphorus levels on egg weight and egg shell quality in laying hens. Poultry Sci. 59:1480-1484.

Owings,W.J., J.L. Sell, and S.L. Balloun. 1977. Dietary phosphorus needs of laying hens. Poultry Sci. 56:2056-2060

Park, K.S. 1986. Refining Egg Specific Gravity Measurement Procedures. MS Thesis. Animal Science Department, Michigan State University, East Lansing. Pages 64.

Paul, H.S. and D.C. Snetsinger. 1969. Dietary calcium and phosphorus and variations in plasma alkaline phosphatase activity in relationship to physical characteristics of eggshells. Poultry Sci. 48:241-251.

Perez, P., and M. Dela Osa. 1987. Effect of different levels of calcium and phosphorus on the production and quality of the eggs of White Leghorn hens. Cuban J. of Agri. Sci. 21:81-84.

Perkin-Elmer. 1982. Analytical methods for atomic absorption spectrophotometry. Norwalk, Connecticut.

Peterson, A.J. and R.H. Common. 1971. Progesterone concentration in peripheral plasma of laying hens as determined by competitive protein-binding assay. Can. J. Zool. 49:599-604.

Peterson, A.J. and R.H. Common. 1972. Estrone and estradiol concentrations in peripheral plasma of laying hens as determined by radioimmunoassay. Can. J. Zool. 50:395-404.

Rabon, H.W. and D.A. Roland, Sr. 1985. Solubility comparison of limestone and oyster shells from different companies and the short term effect of switching limestone varying in solubility in egg specific gravity. Poultry Sci. 64 (suppl. 1): 37 (Abst.).

Ralph, C.L. and R.M. Fraps. 1959. Long term effects of diencephalic lesions on the ovary of the hen. Am. J. Physiol. 197:1279-1283.

Rao, K.S. and D.A. Roland, Sr. 1989. Influence of dietary Ca level and particle size of Ca-source on in vivo Ca-solubilization by commercial Leghorns. Poultry Sci. 68:1499-1505.

Rao, K.S., D.A. Roland, Sr. and F.J. Hoerr. 1992. Response of early- and late-maturing commercial leghorn pullets to low levels of dietary phosphorus. Poultry Sci. 71:691-699.

Reichmann, K.G. and J.K. Connor. 1977. Influence of dietary calcium and phosphorus on metabolism and production in laying hens. Br. Poult. Sci. 18:633-640.

Reid, B.L. and C.W. Weber. 1976. Calcium availability and trace mineral composition of feed grade calcium supplements. Poultry Sci. 55:600-605.

Riddell, C., C. F. Helmoboldt, E. P. Singsen, and L. D. Mattersen, 1968. Bone pathology of birds affected with cage layer fatigue. Avian Dis. 12:285-296.

Riddell, C., 1989. Cage layer fatigue. A review. Proc. 38th Western Poultry Conf., Tempe, Arizona. Pages 87-88.

Roland, Sr. D.A., D.R. Sloan, H.R. Wilson, and R.H. Harms. 1973. Influence of dietary calcium deficiency on yolk and serum calcium, yolk and organ weights and other selected production criteria of the pullet. Poultry Sci. 52:2220-2225.

Roland, Sr. D.A., and R.H. Harms. 1973. Calcium metabolism in the laying hen. 5. Effect of various sources and size of calcium carbonate on shell quality. Poultry Sci. 55:369-372.

Roland, Sr. D.A., D.R. Sloan, and R.H. Harms. 1974. Effect of various levels of calcium with and without pullet sized limestone on shell quality. Poultry Sci. 53:662-666.

Roland, Sr. D.A. and R.H. Harms. 1976. The influence of feeding diets containing different calcium-phosphorus ratios on the laying hen. Poultry Sci. 55:637-641.

Roland, Sr. D.A. 1979. Factors influencing shell quality of aging hens. Poultry Sci. 52:774-777.

Roland, Sr. D.A. 1986. Eggshell quality. IV: Oystershell versus limestone and the importance of particle size or solubility of calcium source. World's Poultry Sci. J. 42:166-171.

Roland, Sr. D.A. 1988. Eggshell problems: Estimates of incidence and economic impact. Poultry Sci. 67:1801-1803.

Roush, W.B., M. Mylet, J.L. Rosenberger, and J. Derr. 1986. Investigation of calcium and available phosphorus requirements for laying hens by response surface methodology. Poultry Sci. 65:964-970.

Ruschkowski, S.R. and L.E. Hart. 1992. Ionic and endocrine characteristics of reproductive failure in calcium-deficient and vitamin D deficient laying hens. Poultry Sci. 71:1722-1732.

Salman, A.J., M.S. Ali, and J. McGinnis. 1969. Effect of level and source of phosphorus and different calcium levels on productivity and phosphorus utilization by laying hens. Poultry Sci. 48:1004-1009

Scanes, C.G., S. Harvey, J. Marsh, and D. King. 1984. Hormones and growth in poultry. Poultry Sci. 63:2062-2074

Scott, M.L., S.J. Hull, and P.A. Mullenhoff. 1971. The calcium requirement of laying hens and effects of dietary oystershells upon eggshell quality. Poultry Sci. 50:1055-1063.

Sell, J.L. 1979. Phosphorus requirement of laying hens: A basic approach. Feed Management. 30:24

Senior, B.E. 1974. Radioimmunoassay of oestrone and oestradiol in the peripheral plasma of the domestic fowl in various physiological states of hypophysectomized and ovariectomized fowls. Acta Endocrinol. 75:133-140.

Senior, B.E. and F.J. Cunningham. 1974. Oestradiol and luteinizing hormone during the ovulatory cycle of the hen. J. Endocrinol. 60:201-202.

Shahabi, N.A., H.W. Norton, and A.V. Nalbandov. 1975. Steroid levels in follicles and the plasma of hens during the ovulatory cycle. Endocrinology. 96:962-968.

Shodono, M., T. Nakamura, Y. Tanabe, and K. Wakabayashi. 1975. Simultaneous determinations of oestradiol 17 B, progesterone and lutenizing hormone in the plasma during the ovulatory cycle of the hen. Acta Endocrinologica. 78:656-573.

Simkiss, K. 1961. Calcium metabolism and avian reproduction. Biol. Rev.. 36:321-367.

Simkiss, R., 1967. Calcium in reproductive physiology. A comparative study of Vertebrate. Chapman and Hall Ltd., London.

Sloan, D.R., D.A. Sr. Roland, and R.H. Harms. 1974. Circadian rhythms of serum calcium in hens, and the relationship of serum calcium to shell quality. Poultry Sci. 53:2003-2009.

Soares, Jr. J. H., 1984. Calcium metabolism and its control. A review. Poultry Sci. 63:2075-2083.

Sommerville, B.A., S. Harvey, and A. Chadwick. 1983. Early changes in the adaptation to a low calcium diet in the chick. Calcif. Tissue Int. 35:339-343.

Sommerville, B.A., R. Swaminathan, and A.D. Care. 1978. A comparison of the effects of dietary calcium and phosphorus deficiency on the in vitro metabolism of 25-hydroxycholecalciferol in the chick. Brit. J. Nutr. 39:411-414.

Sooncharernying, S. and H.M. Edwards, Jr. 1989. Effect of dietary calcium and phosphorus levels on ultra-filterable calcium and dialyzable phosphorus in the laying hen. Poultry Sci. 68:719-723.

Spanos, E., D. Barrett, I. MacIntyre, J.W Pike, E.F. Safilian, and M.R. Haussler. 1978. Effect of growth hormone on vitamin D metabolism. Nature (London) 273:246-247.

Speers, L.G.M., D.Y.E. Perey, and D.M. Brown. 1970. Effect of ultimobranchialectomy in the laying hen. Endocrinology. 87:1292-1297.

Summers, J.D., R. Grandhi, and S. Leeson. 1976. Calcium and phosphorus requirements of the laying hen. Poultry Sci. 55:402-412.

Swaminathan, R., B.A. Sommerville, and A.D. Care. 1977. The effect of dietary calcium on the activity of 25,hydroxycholecalciferol-1α- hydroxylase and calcium absorption in vitamin complete chicks. Br. J. Nutr. 38:47-54.

Taher, A.I., E.W. Gleaves, and M.M. Beck. 1986. Effect of serial blood sampling on interpretation of estradiol 17-ß involvement in laying hen calcium metabolism. Comp. Biochem. Phys. 84A:715-718.

Talmage, R.V., J.H. Roycroft, and J.J.B. Anderson. 1975. Daily fluctuations in plasma calcium phosphate and their radionuclide concentrations in the rat. Calcif. Tiss. Res. 17:19102.

Tanaka, K., M. Kamiyoshi, and M. Kawashima. 1976. Uptake of [^{3}H]progesterone by the anterior lobe of pituitary and some other tissues of the hen during an ovulatory cycle. Acta Endocrinologica. 82:47-51.

Taylor, T.G. 1965. Dietary phosphorus and eggshell thickness in domestic fowl. Br. Poult. Sci. 6:79-87.

Taylor, T. G., A. William, and J. Kirkley, 1965. Cyclic change in the activities of plasma acid and alkaline phosphatase during eggshell calcification in the domestic fowl. Can. J. Biochem. 43:451-457.

Taylor. T. G. 1970. How an eggshell is made. Sci. Am. 222:88-95.

Tsang, C.P.W. and A.A. Grunder. 1985. Prepubertal plasma estradiol and total calcium levels in the strains of white leghorns in relation to eggshell quality. Archiv fur Geflugelkunde. 49:12-15.

Tsang, C.P.W., A.A. Grunder, H. Soares, and R. Narbaitz. 1988. Effects of cholecalciferol or calcium deficiency on oestrogen metabolism in the laying hen. Br. Poult. Sci. 29:753-759.

Urist, M.R., O.A. Schjeide, and F.C. McLean. 1958. The partition and binding of calcium in the serum of the laying hen and of the estrogenized rooster. Endocrinology. 63:570-585.

Vandepopuliere, J.M. and J.J. Lyons. 1992. Effect of inorganic phosphate source and dietary phosphorus level on laying hen performance and eggshell quality. Poultry Sci. 71:1022-1031.

Van de Velde, J.P., F.C. Van Grinkel, and J.P.W. Vermeiden. 1986. Patterns and relationships of plasma calcium, protein, and phosphorus during the egg laying cycle of the fowl and the effect of dietary calcium. Br. Poult. Sci. 27:421-433.

Warren, D.C. and H.M. Scott. 1935. The time factor in egg formation. Poultry Sci. 14:195-207.

Wasserman, R.H. and G.F. Combs, Jr. 1978. Relation of vitamin D dependent intestinal calcium binding protein to calcium absorption during the ovulatory cycle in Japanese quail. Proc. Soc. Exper. Biol. Med. 159:286-287.

Williams, J. 1990. Differences in performances and endocrinological variables in two strains of chickens. In Control of Fertility in Domestic Birds, Tours, France. 2-4 July. Paris, France; Institut National de la Recherche Agronomique Colloques de l'INRA. 54:21-32.

Wilson, S.C., and P.J. Sharp. 1975. Changes in plasma concentrations of LH after injection of progesterone at various times during the ovulatory cycle of the domestic hen (Gallus domesticus). J. Endocrinol. 69:93-102.