EGGSHELL QUALITY IN THE LAYING HEN AS RELATED TO PROGESTERONE AND ESTRADIOL

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CHAPTER I

LITERATURE REVIEW

Introduction

One of the major economic problems of the poultry industry is that eggshell quality steadily deteriorates after the first few months of egg production. This decline in eggshell quality is detected in about 25% of a flock. It has been estimated that 5 to 7% of all eggs produced in the United States are lost due to cracked or broken eggs (Petersen, 1965).

Many factors interact in the control of eggshell quality (Wolford and Tanaka, 1970; Petersen, 1965). Eggshell thickness is a hereditary factor in which significant improvement can be attained, but apparently only at the expense of an equally significant decrease in egg production (Van Tijen, 1977). Improved nutritional and management practices have not entirely solved the problem.

Attention is now being directed in the investigation of the biochemical and physiological systems that may influence eggshell quality. Presently it is not known how the reproductive hormones of the hen fluctuate during an egg production cycle or how they might relate to eggshell quality. These hormone concentrations are being measured in the blood and tissues of the hen. The hormone concentrations are being related to compounds which can directly influence calcium and phosphorus absorption and metabolism, and thus eggshell quality. Calcium binding

protein in the intestine and uterus and Vitamin D metabolites in the kidney and bloodstream are examples of these compounds.

The objective of this study was to measure the concentration of estrogen and progesterone in the plasma of the laying hen through a portion of the egg production cycle and determine if relationships occur between these hormones and eggshell quality.

Reproductive System in Avian Females

Anatomy and Function

The functional reproductive system of the female consists of the left ovary and left oviduct. Embryologically, two ovaries and oviducts are present, but the organs of the right side do not normally develop. The ovary consists of an outer cortex and an inner medulla. The cortex of the producing hen contains a number of developing follicles of various sizes and a large number of small and microscopic ova (Pearl and Schoppe, 1921).

The oviduct is comprised of several distinct areas which include the infundibulum, magnum, isthmus, shell gland and vagina. The infundibulum is about 9 cm long and is the thin, fimbrated tissue that engulfs the ovum after ovulation. The ovum passes to the magnum (53 cm long) where most of the albumen of the egg is formed. Peristaltic movements of the magnum force the ovum into the isthmus (10 cm long). Here the inner and outer shell membranes are formed. The ovum passes from the isthmus to the red isthmus where the initiation of shell calcification occurs. In the shell gland or uterus (10 cm long), water and salts are added to the albumen, the shell is deposited around the ovum, and pigments (porphyrin) are added to the eggshell. The egg passes to the

vagina which aids in the expulsion of the egg (Warren and Scott, 1935).

As soon as the ovum is entirely engulged by the infundibulum, the ovum appears to act as a stimulus to the entire oviduct to begin contractions (Warren and Scott, 1935). Production of the albumen, shell membranes and shell is thought to be a reflex action to the passage of an object through the oviduct. Sympathetic and parasympathetic nerves may play a minor role in albumen formation.

Laying Cycle

It takes an average of 13 minutes for infundibulum to pick up the ovum after ovulation. The ovum will stay in the infundibulum from 15 to 30 minutes. The average time spent in the rest of the tract is as follows: magnum, 2 to 3 hours; isthmus, 1¼ to 2 hours; shell gland, 21 to 22 hours; and vagina, 15 minutes. The total time from ovulation to oviposition ranges from 24 to 28 hours (Warren and Scott, 1935).

Chickens are continuous breeders that mate and lay eggs at all times of the year. Commercial egg laying strains will not incubate their eggs. Hens will lay eggs on successive days, to form a clutch, and then stop laying for one or more days before egg laying is resumed. The first egg in a clutch is generally laid early in the morning. Succeeding eggs in a clutch are laid 24 to 28 hours apart so that the eggs are laid progressively later each subsequent day. The last egg in a clutch is generally laid in mid or late afternoon (Atwood, 1929).

Prolific hens may lay 250 to 270 eggs per year. The peak of production generally occurs at about six months of age. At this time, 90% of a flock should be laying each day, and individual hens will have clutches of 20 or more eggs. At 12 to 13 months of egg, only about 75%

of the hens will lay each day. Clutches will be smaller, containing 5 to 10 eggs.

Approximately 15 to 90 minutes after oviposition, ovulation of the next egg in a clutch will occur. Premature oviposition seems to have no effect on the time of ovulation. Ovulation of the next egg of a clutch will occur at the time it would have had premature oviposition not occurred (Warren and Scott, 1935).

The interval in hours between oviposition of successive eggs in a clutch is greater in hens with small clutches compared to hens with large clutches. The interval between the first two eggs and the last two eggs in a clutch are greater than for intervening eggs, regardless of the size of the clutch (Heywang, 1938). Prolific hens have shorter intervals between ovipositions than low producers. The differences in production appear to be due to differences in the length of time the ovum spends in the uterus. The ovum of prolific hens spend less time in the eggshell gland than those of low producers. The time between oviposition and ovulation appears to be equal for both groups, as is the time spent in the magnum and isthmus (Warren and Scott, 1935).

Ovulation

Immature ova grow rapidly as pullets reach sexual maturity and require about 9 to 10 days to mature. About seven days prior to the first ovulation, the weight of the yolk and ova increase linearly about 16-fold (Warren and Conrad, 1939). There is a graduation in maturation of developing follicles called a hierarchy. This hierarchy usually involves four to six follicles, with longer hierarchies generally visible in more prolific hens (Conrad and Scott, 1938). The hens endocrine system must

thus ensure growth of several ovarian follicles at the same time. The follicles must reach ovulatory size at closely spaced intervals and the most mature follicle is ovulated at a closely regulated time. Regulation of the hierarchy is not understood. Follicular growth may be controlled by release of follicle stimulating hormone (FSH) or lutenizing hormone (LH), or growth may be controlled by some factor produced by the ovary itself. Mitchell (1967) was able to maintain a normal follicular hierarchy in hypophysectomized hens by administration of FSH alone.

Endocrine Control

Ovulation occurs when the stigma of the follicle ruptures (Phillips and Warren, 1937). It appears to be caused by the release of LH from the anterior pituitary. Heald (et al., 1968) observed there was a significant decrease in the concentration of LH in the pituitary 4 to 8 hours prior to ovulation. Several studies (Senior and Cunningham, 1974; Furr et al., 1973; Etches and Cunningham, 1977; Shahabi et al., 1975; Shodono et al., 1975; Lague et al., 1975) have found increased blood plasma concentrations of LH 4 to 8 hours prior to ovulation. Maximal plasma concentrations of LH have been determined to be 1.7 to 5 mg/ml, while basal levels have averaged about 1.0 mg/ml. If an egg is not ovulated, plasma LH concentrations remain at basal concentrations.

FSH may augment the ovulatory effect of LH (Kamiyoshi and Tanaka, 1972). Injections of both LH and FSH, at dosages at which neither alone can cause ovulation, will cause premature ovulation. FSH will also increase the incidence of premature ovulation of FSH if given either one hour before or one hour after an LH injection.

Progesterone induces premature ovulation when injected into certain

regions of the hypothalamus or the caudal neostriatum. However, progesterone does not influence ovulation when injected into other parts of the forebrain or the pituitary (Ralph and Fraps, 1960). Thus progesterone probably induces ovulation by stimulating secretion of releasing factors of the brain which act upon the pituitary hormones.

The ability of either progesterone, LH or LH releasing hormone (LH-RH) to induce premature ovulation seems to be dependent on the stage of maturation of the ovum (Etches and Cunningham, 1976). It was also indicated that LH secretion stimulates progesterone secretion and vice-versa. This suggests that part of the function of progesterone is to maintain the surge of LH that will induce ovulation.

Both LH and progesterone reach maximal plasma concentrations 4 to 7 hours before ovulation (Williams and Sharp, 1978; Lague et al., 1975; Shodono et al., 1975; Furr et al., 1973). The rise in LH may precede the increase in plasma progesterone (Lague et al., 1975) or plasma concentrations of progesterone and LH may increase simultaneously (Furr et al., 1973).

Wilson and Sharp (1976) demonstrated that LH release in ovariectomized hens required the combined actions of estrogen and progesterone in a two phase process. First, there is a primary phase which requires the presence of both estrogen and progesterone. A definite estrogen: progesterone ratio is required to prime the LH feedback mechanism as a change in concentration of either hormone will modify LH response. The second phase is the inductive phase which depends on the incremental change in plasma progesterone concentration to stimulate LH secretion. Thus, this study also supports the hypothesis that increasing plasma concentrations of progesterone cause the preovulatory discharge of LH.

Estrone, estradiol 17β, estradiol 17α (Common et al., 1965) and estriol (Mathus and Common, 1967) are the principal estrogens in the urine, blood and ovaries of the laying hen. Brown et al. (1978) has demonstrated the existence of several other estrogenic steroids in the plasma of the laying turkey. Estrone and estradiol concentrations increase significantly in the plasma 4 to 7 hours prior to ovulation and are unaltered on days when ovulation does not occur (Senior, 1974; Graber and Nalbandov, 1976; Peterson and Common, 1972). This increase in plasma estrogen is concurrent with the increase in plasma LH and progesterone (Shodono et al., 1975; Lague et al., 1975) and androgens (Peterson et al., 1973; Furr and Thomas, 1970; Shahabi, et al., 1975; Etches and Cunningham, 1977) that occur 4 to 7 hours prior to ovulation. Senior and Cunningham (1974), however, indicated that the rise in estrogen concentration precedes that of LH by about two hours.

While most studies indicate the presence of one peak of estrogen in the plasma, Shadono et al. (1975) has reported two peaks of estrogen. The first occurred at 24 hours prior to ovulation and the second peak was at 4 to 5 hours before ovulation. Peterson and Common (1972) also indicate two peaks of estradiol; at 22 to 18 hours and 6 to 2 hours prior to ovulation. Four peaks of total estrogens in the plasma have been indicated by Graber and Nalbandov (1976). They occur at 23 hours after ovulation and 22, 13 and 8 hours before ovulation. Each increase in plasma estrogen concentration may possibly have a specific function in the formation of the egg and control of ovulation.

Eggshell

Formation

The eggshell is composed of three layers. The innermost layer consists of two proteinaceous membranes and next are the true shell and a proteinaceous cuticle (Stewart, 1935). The formation of the shell originates in the red or mid-isthmus with the deposition of small surface granules on the outer shell membranes. In the mid-isthmus the membrane has about 270 granules/mm^2, ranging in size from 1-10 μm (Stemberger et al., 1977). The number of granules increase and then decrease as the granules aggregate. The granules are believed to serve as the nuclei for deposition of the secretions of the shell gland.

The true shell is composed of the mammillary layer, which is primarily composed of organic material, and the palisade layer, which is primarily inorganic. The mammillae originate in the shell gland where the mammillary cores are deposited and nucleation of the calcite crystals takes place (Stewart, 1935). The egg remains in the shell gland about 20 hours. During the first five hours, calcium carbonate deposition increases gradually to a rate of 300 mg/hour. This rate will then remain constant until about two hours prior to oviposition (Burmester, 1940). Water movement into the albumen also occurs. The addition of water to the albumen stretches the shell membranes so that the distance between the tips of the mammillae increase (Hansen, 1934). At the same time, the calcite crystals radiate from the mammillary core and increase in size. The crystals anchor the shell into the shell membrane, extend to crystals from other mammillary cores and extend upward to the shell surface. Pores are formed where the crystals do not completely grow

together.

Calcium Metabolism

Calcium moves across the shell gland at a rate of 100 to 150 mg/hour during the last 15 hours of shell formation. This transport is believed to be active (Hurwitz et al., 1970). Calcium binding protein, which mediates active calcium transport in the intestine, has been found in the shell gland (Corradino et al., 1968). The amount of total calcium in the shell gland appears to move against a gradient because shell gland fluid can contain as much as 26 mmoles/liter of calcium while blood serum has only 6 to 7 mmoles/liter (ElJack and Lake, 1967).

The shell gland does not store very large amounts of calcium so calcium must be continuously replenished from the blood. Nearly all blood calcium is in the plasma. Blood calcium exists in a non-diffusible protein bound form and as diffusible ionic calcium (Thaeler, 1979). The two forms function in a dynamic equilibrium. Serum calcium concentrations rise from 10 mg/100 ml blood to 16 to 30 mg/100 ml during the 10 days before a pullet starts to lay. The increase only occurs in the non-filterable calcium form and is caused by the production which is under the control of estrogen, of a complex of phosphoproteins which bind large quantities of calcium (Thaeler, 1979).

Control of Calcium Absorption

Since shell formation requires 100 to 150 mg of calcium per hour and the laying hen only has a blood concentration of 20 to 30 mg/100 ml blood, the blood would be depleted of calcium in less than 15 minutes if it were not continuously replenished. Calcium absorption from the

intestine (Hurwitz and Bar, 1965) and resorption of calcium from bones (Taylor, 1965) are active processes. The amount of calcium absorbed and the efficiency of absorption and retention varies with the physiological state of the hen.

About 10 days before a pullet starts to lay, the retention of dietary calcium and phosphorus increases markedly (Halnan, 1925). Most of the retained mineral appears to be incorporated into bone, as the skeletal weight increases by 20% at this time. The improved mineral retention is caused by the synergistic action of estrogen and androgen (Common et al., 1948; Common et al., 1953).

Calcium binding protein activity in the intestine is low before the initiation of egg laying, but reaches a normal concentration after the first egg is laid (Bar and Hurwitz, 1972). If egg production is interrupted for a short time by Nicarbazin, a drug which stops egg production but does not interfere with secretion of gonadal hormones, calcium binding protein drops to a concentration similar to nonlayers. Activity will return to 75% of its original level after the first oviposition again occurs. The calcium binding protein decreased in spite of maintenance of estrogen dependent parameters. This suggests that the gonadal hormones do not directly affect the induction of calcium binding protein (Bar and Hurwitz, 1972).

The percentage of calcium absorption is increased during eggshell deposition (Hurwitz and Bar, 1965) along most of the intestine, especially the upper jejunum (Hurwitz et al., 1973). Phosphorus absorption is also increased, but to a lesser degree (Hurwitz and Bar, 1965). There is no change in intestinal binding protein at this time (Bar and Hurwitz, 1972; Montecuccoli et al., 1977). It has been suggested that

there are actually two regulatory mechanisms for intestinal calcium absorption (Hurwitz et al., 1973). One regulatory mechanism acts in the duodenum to respond to long term changes in calcium needs such as the onset of egg production. The other mechanism, which is along most of the small intestine, responds to short term changes in calcium demands such as shell formation.

Most of the increase is skeletal weight during the 10 days prior to sexual maturation is due to the formation of medullary bone. It occupies the marrow cavities of bones, growing from the endosteal surface of the bone in the form of fine, interlacing spiculas (Kyes and Potter, 1934). Taylor (1965) argues that medullary bone is mobilized to provide calcium for eggshell formation regularly, even when dietary supplies of calcium are adequate. Bone that is resorbed during shell calcification at night is resynthesized during the day when dietary calcium supplies are increased. The process of medullary and cortical bone breakdown and resynthesis is regulated by the parathyroid.

Hurwitz and Bar (1969) believe the function of medullary bone is supplying calcium to the eggshell is overemphasized. In a study using ^{45}Ca, there was no significant quantitative change in bone calcium mass during a laying cycle (Hurwitz, 1964; Hurwitz and Bar, 1969b). Laying hens selected for either heavy or light shells did not have significant differences in bone turnover rate (Hurwitz and Bar, 1967). If shell formation is not occurring, calcium absorption is about 40% on a diet adequate in calcium. During egg calcification, calcium absorption increases to 70% (Hurwitz and Bar, 1969). If one assumes a constant flow of nutrients through the intestines, the need for calcium during shell formation can be met by increased absorption. However, if the diet is

calcium deficient, maximal absorption is inadequate to meet calcium needs. Calcium will then be mobilized from the bone (Hurwitz and Bar, 1969). In long term calcium deprivation (30 days at 1.7% calcium), there will be a significant increase in calcium binding protein to increase calcium absorption so that neither egg production nor plasma calcium will be lessened (Bar and Hurwitz, 1972). In even more severe deficiencies the hen will produce thinner eggshells and finally stop production.

The increase in efficiency of calcium absorption is believed to be regulated by metabolites of Vitamin D. Under conditions of adaptation to low intakes of calcium and phosphorus, greater 1,25 dihydroxycholecalciferol is produced by the kidney. This compound then accumulates in the intestinal mucosa. Calcium binding activity will also increase, resulting in increased absorption of calcium and phosphorus (Edelstein et al., 1975). The increased calcium binding activity was shown to be due to intestinal calcium binding protein which changes in direct proportion to the change in calcium absorption (Morrissey and Wasserman, 1971). Calcium binding protein in both the intestine and uterus has been shown to be significantly increased by vitamin D_3 administration in the hen (Corradino, 1968) and the Japanese quail (Bar et al., 1976).

As stated earlier, the improved calcium and phosphorus absorption and retention associated with sexual maturity of a pullet appears to be caused by the synergistic action of estrogen and androgens. The action of these hormones is mediated through 1,25 dihydroxyvitamin D_3 which in turn can control increases in intestinal calcium binding protein, and thus increased calcium absorption (Friedlander et al., 1977; Bar and Hurwitz, 1979).

Mature female Japanese quail and chickens have predominantly 25 hydroxyvitamin D_3 1-hydroxylase activity in their renal tissue. The mature male has predominantly 25 hydroxyvitamin D_3 24-hydroxylase activity. When estrogen was given the male or female, the 1-hydroxylase was markedly stimulated, and the 24-hydroxylase suppressed. Both estrogen and testosterone are required to achieve the same effect in the immature, castrate male (Tanaka et al., 1976). Other hormones such as FSH, cortisone, testosterone alone, and progesterone produced little or no change in 25-hydroxyvitamin D_3 1-hydroxylase activity. Parathyroid hormone can stimulate 1-hydroxylase (Castillo et al., 1977). The effects of estrogen and parathyroid hormone administered together are additive, suggesting they function by different mechanisms.

Kenny et al. (1974) observed that kidneys from all hens with an egg in the oviduct had enhanced 1,25 hydroxyvitamin D_3 production. However, Tanaka et al. (1976) were unable to observe this response.

Tanaka et al. (1978) attempted to elucidate the control mechanisms of 25 hydroxyvitamin D_3 1-hydroxylase using castrate male chickens. It was found that testosterone or progesterone administered alone or together cannot significantly affect 1-hydroxylase. If estradiol is given with both testosterone and progesterone, a synergistic effect is achieved and greater concentrations of 1-hydroylase activity are present in the kidney. Concurrently, a three-fold increase in plasma 1,25 dihydroxyvitamin D_3 occurs. It is probable that it is through this mechanism that estradiol and progesterone can control calcium metabolism and, thus, eggshell quality.

Eggshell Quality

Evaluation Techniques

The strength of the egg has been directly measured by methods that involve crushing (Stewart, 1936; Godfrey, 1949) impact (Mueller, 1957) and puncturing. Wilson et al. (1968) counted the number of emitted beta particles that were returned from a given area of shell and found significant correlations between counts received and resistance of the shell to impact. Frank et al. (1964) indicated that not more than 60% of the variation in crushing strength could be explained by differences in shell thickness, specific gravity, percent shell, and shell weight. None of the chemical components of the shell can account for a large percentage of the variation in resistance to shell failure (Frank et al., 1965). The shape of the egg appears to be independent of shell thickness, but can explain 15 to 33% of the variability in crushing strength which remains after shell thickness has been considered (Richards and Swanson, 1965).

Specific gravity has long been used to estimate eggshell quality (Hammerle, 1969) and has often been a preferred method because it is rapid, practical, inexpensive and does not require that the eggshell be broken. Potts (1974) reported correlation coefficients between shell thickness and specific gravity which ranged from .56 to .88, depending on the strain of birds from which eggs were obtained. Correlation coefficients between breaking strength and specific gravity ranged from .72 to .80. He also determined that although shell thickness, specific gravity and shell deformation values for brown egg strains were poorer than for white egg strains, the breaking strength of shells from brown

egg strains was higher. Therefore, thinner shells are not necessarily weaker and more than one criteria should be used to evaluate eggshell strength.

Factors That Relate to Eggshell Quality

Factors that affect eggshell quality have been reviewed by Petersen (1965) and Wolford and Tanaka (1970). Some of the factors that relate to eggshell quality are as follows:

Age of Hen. Eggshell quality has been reported to decrease with the age of the hen (O'Neil and Rae, 1952; Mueller et al., 1960; Brunson and Godfrey, 1953). Shell quality will improve when a hen resumes egg production after a molt (Nobles, 1966; Berg and Bearse, 1947).

Environmental Temperature. Eggshell quality will decrease when temperatures, either constant or fluctuating, are over 32 C (de Andrade et al., 1976; de Andrade et al., 1977; Huston and Carmon, 1961). Mueller (1959, 1961) found a significant decrease in shell thickness when hens were subjected to high temperatures even though the hens exhibited a greater level of calcium retention than hens in lower temperatures.

Hatchability. Specific gravity has been related to hatchability in hens (Godfrey and Jaap, 1949; Mussehl and Halbersleben, 1923) but not in turkeys (Brunson and Godfrey, 1953). McDaniel et al. (1979) found that there is a sharp decline in hatchability of eggs with a specific gravity less than 1.080. Thus, in young flocks where the specific gravity of eggs averages above 1.080, it is difficult to show a relationship between specific gravity and hatchability. In older flocks,

where large numbers of eggs fall below 1.080, there is a strong, positive relationship between specific gravity and hatachability.

Shell Structure. Most of the changes responsible for differences in shell breaking strength apparently take place in the palisade layer of the shell. As the palisade decreases in thickness and density, there is a predictable decrease in the strength of the eggshell. The thickness of the mammillary layer of the shell remains almost constant regardless of the strength of the shell (Meyer et al., 1973). However, the mammillary cores from a large percentage of weak eggshells are abnormally irregular and diffuse (Robinson and King, 1970).

Shell Gland Ultrastructure. Huntley (1978) used scanning and transmission electron microscopy to study the surface subsurface ultrastructure of shell glands from hens producing either good or poor eggshells. The subsurface ultrastructure appeared to be the same regardless of the eggshell quality. The surface of the shell gland from hens producing good quality eggshells had a larger quantity of clumped cilea and spheres of material. The spheres were not composed of calcium.

Genetics. Shell quality appears to be an inheritable trait (Taylor and Lesner, 1939). There appears to be a significant difference among breeds of hens in shell thickness, but differences among strains within breeds are not apparent (King and Hall, 1955). Van Tijen (1977) determined that shell quality can be improved by selective breeding. He found that after four generations of selecting exclusively for improved shell quality, a significant improvement in quality occurred as compared to the strain of hens selected for productivity, egg weight, shell quality and egg internal quality. However, the improvement in shell quality

was gained at a loss of 10 to 11% in egg production. When the two selected stains were crossed, there was no heterosis effect in improvement of eggshell quality (Van Tijen, 1977b).

Calcium Binding Protein. Bar and Hurwitz (1973) found no difference in the activity of intestinal and uterine calcium binding protein between hens that produced eggs with heavy or light shells, although there was a trend towards having higher activity associated with heavier eggshells. Uterine calcium binding protein was not correlated with the amount of calcium secreted by the shell gland or with eggshell thickness.

Calcium and Phosphorus. It has long been recognized that reduced calcium intake has detrimental effect on eggshell quality (Wolford and Tanaka, 1970). Hens fed diets that contained 2.43%, 1.24% or 0.2% calcium had significant differences in egg production and eggshell strength with characteristics reduced for diets with the lowest calcium intake, or vice versa, were followed by significant changes in egg production in 4 to 8 days and significant change in eggshell strength in one day (Wehring, 1964). Eggshell quality improves in hens with dietary levels of calcium up to 4.0 or 4.5% (Roland, 1974).

When hens are given the opportunity to regulate food and calcium intake, they can control these two parameters independently. There will be a characteristic diurnal pattern of calcium intake, which is dependent on whether or not a hen is forming an egg on that day. Calcium intake is very low on days when there is no egg formation and very high on days when egg formation occurs. Feed intake is much less variable, but tends to be higher on days when eggshell formation occurs. The stimulus for calcium intake appears to originate from the act of ovulation

and not from shell formation. It appears that endocrine changes near ovulation influence calcium intake (Hughes, 1972). Young hens can successfully adjust both calcium and phosphorus intake to maintain egg production, egg weight and specific gravity when given the rations of differing calcium or phosphorus intakes (Holcombe et al., 1975; Holcombe et al., 1976).

The calcium: phosphorus ratio can influence serum calcium and the specific gravity of eggs. A ratio of 2:1 (Ca:P) caused a decrease in specific gravity, while a ratio of 9:1 caused a significant increase (Roland et al., 1976).

Increasing the phosphorus levels of a ration from about 0.3 to 0.7% did not significantly affect shell thickness, while lower levels did result in thinner eggshells (Crowley et al., 1963; Walter and Aitken, 1962). Hinners et al. (1963) reported no significant change in shell thickness or egg production when the amount of phosphorus in the diet was increased from .705 to 1.018%.

Time of Oviposition. The later in the afternoon the egg was laid, the greater the specific gravity (Roland et al., 1973; Roland and Harms, 1974; Wilhelm, 1940). They believe, though, that this is not entirely due to the egg remaining in the uterus longer. Eggs laid in the afternoon are rounder and weigh less than eggs laid in the afternoon (Roland and Harms, 1974; Roland, 1978). Rounder eggs tend to have greater specific gravity (Essary et al., 1978; Roland, 1978). Thus with a given amount of shell, a thicker shell can be produced on a rounder, smaller egg than a large, oval egg.

CHAPTER II

MATERIALS AND METHODS

Development of Extraction Techniques

Introduction

Quantification of steroid hormones in small volumes of plasma from avians is a relatively new technique. Radioimmusoassay procedures developed for other species have been used even though the assays have frequently not been fully validated for use with the avian. The laying hen has a very high concentration of blood lipids compared to other vertebrate animals; Balnave (1971) determined total lipids in active layers to be 2548 ± 49 mg per 100 ml of blood. The high concentration of lipids in the blood appears to interfere with many assay procedures originally developed for other species.

Another factor that complicates the development of assays for plasma steroids is that the avians appear to have several different estrogens in the plasma in addition to estrone, estradiol and estriol which are present in most mammals. Brown et al. (1978) has identified two additional estrogen compounds in turkey plasma and has tentatively identified three more. As these estrogens may all possibly cross react with antibodies in radioimmunoassays currently being used for mammals, careful purification of the estrogens of interest is required.

Evaluation of Extraction and Purification Methods

All solvents used in the extraction and assay, except diethyl ether, were freshly distilled prior to use. A new can of diethyl ether was opened for each replicate of samples quantified.

Estradiol 17β is extracted from porcine and bovine plasma in our laboratory with 3 ml of benzene added to 1 ml plasma. The extracts are vortexed for 90 seconds and the benzene is transferred to clean tubes (Hallford et al., in press). When this method was used for hen plasma, three problems became apparent. The first was a technical problem; a solid emulsion frequently formed after the plasma was vortexed with benzene. The second and third problems arose during the validation of the assay. It was determined that the dose response curves of the unknowns were not parallel to the standard curve, and two immunological peaks resulted when an estrogen extract was chromatographed on a 1 x 36 cm Sephadex LH-20 column eluted with chloroform: ethanol (95:5 v:v) (Swanson et al., 1972). The first estrogenic peak was eluted with the ^{3}H estradiol 17β reference and the second was approximately 8 ml later. This demonstrated that the antibody, which was specific for estradiol 17β is cattle and swine, reacted with some other estrogenic compounds in hen plasma.

The standard procedure in our laboratory is to extract progesterone from the plasma of the bovine and porcine species with benzene: hexane (1:2 v:v) (Kittok et al., 1973). Estimates of progesterone concentration using this extraction procedure for hen plasma were compared to those obtained on the same samples after the progesterone was isolated from

the extract using Sephadex LH-20 columns (Swanson et al., 1972). The quantity of progesterone in the extracts from the standard procedure was consistently less than that determined after column chromatography. This indicated that some component in the plasma inhibited the progesterone antisera used in the assay from binding progesterone. However, this substance was apparently removed when the progesterone was purified, so that a part of the inhibition was eliminated. This problem was further compounded by the fact that the dose response curves for either plasma extracts or chromatographed extracts were not parallel to the standard curves and that recovery of mass was only 30 to 60%. Using different ratios of benzene: hexane (1:2, 2:1, 1:3 and 1:4) for extraction did not improve the results.

Since the assays used to quantify progesterone and estradiol in porcine and bovine plasma could not be validated for avian plasma, several other extraction procedures were evaluated. First diethyl ether was used to extract progesterone from the plasma. The first antibody was diluted 1:1500 instead of the usual 1:3000 dilution to determine if enough antibody was being added. The results demonstrated that the dose response curves were not parallel to the standard curve and recovery of mass was very low (30 to 50%).

Monk et al. (1976) described an extraction procedure in which plasma was incubated with diethyl ether for .5 hour in a 40 C water bath after which the solutions were shaken for 15 minutes and frozen. The solvent phase was decanted and evaporated to dryness. The extracts were redissolved in 2.5 ml of methanol: water (7:3 v:v), then incubated for 1 hour in a 40 C water bath and frozen. The extracts were centrifuged for 30 minutes at 1600 xg. The methanol layer was transferred to clean test

tubes and aliquots of the solvent were taken for completion of the assay.

While this method produced a preparation containing less visible fat, the dose inhibition curves were still not parallel. The amount of quenching of the radioactivity in this assay was evaluated to determine if the cause of nonparallelism was due to an alteration in the efficiency of counting the radioisotope. However, the quench observed did not differ significantly between the unknowns and the standards.

A discontinuous florosil column, similar to the one described by Swanson (1970) was evaluated. Plasma progesterone was extracted according to the method described by Monk et al. (1976). The plasma extract was applied to a 1 x 36 cm florosil column and was eluted with 40 ml of chloroform: acetone (7:3 v:v) followed by 40 ml of chloroform: methanol (9:1 v:v). The ^{3}H progesterone was eluted in two peaks; the first peak of progesterone was detected near the midpoint of the chloroform: acetone elution while the second peak of progesterone was detected near the midpoint of the chloroform: methanol elution. This may occur because the progesterone is bound to lipid soluble compounds, some of which are eluted with methanol and others are eluted with acetone. This would indicate that the lipid fraction is not separated from progesterone which made this extraction procedure unacceptable for use with the assay.

Kappauf and van Tienhoven (1972) described an extraction method for progesterone which was used in conjunction with the Monk procedure. Plasma was incubated with .25 to 1.0 ml of 1 N NaOH for 30 minutes, shaken vigorously for 30 minutes, and the extraction was then completed according to the Monk procedure. When the plasma contained low levels of progesterone, the dose response curves of the unknowns appeared to be parallel to the standard curve. However, at higher levels of plasma

progesterone (5-9 ng/ml), the curves were not parallel.

A single antibody radioimmunoassay for progesterone (Kittok et al., 1973) was then investigated. All progesterone assays, except this one, were performed by the double antibody procedure described on page 29. Plasma progesterone was extracted according to Monk et al. (1976). The extracts were incubated with 200 µl of antiprogesterone (#869, donated by Dr. Niswender, at a dilution of 1:2,000 in PBS + gel) for 15 minutes. ^{3}H progesterone (107 C/mM), 5000 dpm, was added. The free progesterone was separated from the bound by adding a charcoal-dextran solution and then centrifuging the solutions at 1600 xg for 10 minutes. Radioactivity was determined in a .5 ml aliquot of the supernatant. Results showed the dose response curves of the unknowns were not parallel to the standard curve, and there was low recovery of mass (30%).

The next extraction procedure evaluated for progesterone was performed as described by Yannone et al. (1964). After plasma was extracted with diethyl ether, the ether phase was transferred to a clean test tube and evaporated to dryness. The extracts were redissolved in 5 ml of 70% methanol and then frozen. After the solutions were centrifuged at 1600 xg for 30 minutes, the solvent phase was transferred to another clean test tube. Heptane (5 ml) was added, the solutions were vortexed, and the heptane phase was discarded. The 70% methanol phase was dried to 2 ml, which resulted in a 40% methanol phase. Diethyl ether (5 ml) was added, the solutions were vortexed, and the ether phase transferred to a clean test tube. Aliquots for the assay were taken from this ether extract. This extraction method produced results that had good recovery of mass, but the dose response curves of the unknowns were still not parallel to the standard curve.

Plasma estrogen was extracted by a method described by Brown et al. (1978). Plasma was vigorously shaken with diethyl ether and frozen overnight. The ether phase was decanted and evaporated to dryness. Toluene (5 ml) and 1 N NaOH (5 ml) were added. The solution was vortexed for 2 minutes and then centrifuged at 260 xg for 10 minutes. The toluene was discarded and 1 ml of saturated carbonate buffer (pH 9.0) was added to the extracts. The extracts were titrated to pH 9 with 20% HCl and diethyl ether (5 ml) was added. After vigorous shaking, the ether phase was transferred to a clean test tube from which aliquots for the assay were taken. The dose response curves of the unknowns were not parallel to the standard curve. An attempt to extract plasma progesterone with this method was done. However, this method extracted phenolic compounds exclusively, so progesterone could not be extracted.

An extraction procedure using silica gel columns (Goldzieher et al., 1961) was used to partially purify both progesterone and estradiol. Plasma steroids were extracted with benzene: hexane (3:1 v:v) and layered onto a 7 mm x 7 cm silica gel column with this same solvent. The steroids were eluted with 3 ml hexane, followed by 9 ml benzene: ether (95:5 v:5) and 7.5 ml ethyl acetate. Each solvent was added just as the last of the previous solvent entered the column bed. The first 2.5 ml fraction of ethyl acetate eluted from the column was collected for steroid quantification. This fraction contained both estradiol and progesterone. The elution pattern for the silica gel column is shown in Table I.

Progesterone and estradiol were quantified in the ethyl acetate fraction and the dose response curves for the unknowns were parallel to the steroid extraction curves. Acceptable recovery of mass (95%), however, was achieved only for progesterone. After several solvents were

TABLE I

ELUTION PATTERN FROM SILICA GEL COLUMNS OF PROGESTERONE AND ESTRADIOL FROM THE PLASMA OF THE LAYING HEN

Solvent Eluted	Fraction Number	Progesterone[a] dpm/2.5 ml	Estradiol[b] dpm/2.5 ml
Hexane	1	0	0
exane plus benzene:diethyl ether (95:5 v:v)	2	0	0
Benzene:diethyl ether (95:5 v:v)	3	9	12
	4	6	12
	5	0	15
Ethyl acetate	6	1400	1690
	7	44	31
	8	6	0

[a] 6600 dpm ^{3}H progesterone was applied to the column.

[b] 13000 dpm ^{3}H estradiol 17β was applied to the column.

tested, diethyl ether was selected for the initial steroid extraction and benzene was chosen to layer the extract onto the silica gel column. This modification improved the recovery of mass of the estrogen which had been added to plasma samples. The columns were flushed with ethyl acetate prior to use to decrease column blank values.

The results of a progesterone assay that used the silica gel purification was compared to that of purification with a Sephadex LH-20 column followed by a silica gel column. This was done to determine whether or not the silica gel extraction was specific for progesterone. The results were different ($P < .05$). The assay that used only the silica gel column gave consistently higher values. It was concluded that the Sephadex LH-20 column removed additional components from the extract. By using the silica gel column first, followed by the Sephadex column, it was believed that a valid assay could be achieved. Progesterone and estradiol could be extracted together since the Sephadex column would separate the two hormones and they could then be assayed separately. Also, by using the Sephadex column, estradiol would be separated from other estrogenic compounds with which the estrogen antibody cross reacted. This two column extraction method was subsequently used to isolate progesterone and estradiol from the plasma of laying hens. Details of this system are described on page 29

General Procedure

Pullets, hybrid H&N Nick Chicks, were purchased at 22 weeks of age. They were housed in a temperature controlled, artificially lighted house in individual laying cages. A lighting regime of 16 hours of light and 8 hours of darkness was used. Water and feed were provided ad libitum.

The layer ration was similar to that used in commercial egg production and met or exceeded NRC nutrient requirements (Appendix Tables VI and VII). Standard management procedures for laying hens in cages were followed. Egg production was recorded daily. Egg weight and egg specific gravity were measured three days each week.

Specific Gravity and Egg Weight Determinations

Specific gravity solutions were made with NaCl and tap water (Handbook of Chemistry and Physics) in 20 gallon plastic pails and kept in a temperature controlled (22 C) room. Solutions were allowed to stabilize for at least five days before being used. The specific gravity of the solutions were checked weekly with a hydrometer by placing a liquid specimen in a glass cylinder so that the meniscus of the hydrometer could be viewed horizontally. All measurements were done by the same person using the same hydrometer (Voisey and Hamilton, 1977). Seven solutions were made, having the following specific gravities: 1.070, 1.075, 1.080, 1.085, 1.090, 1.095, and 1.100.

Eggs were marked with their respective hen number. They were kept in the same room as the specific gravity solutions for about four hours before egg weight and specific gravity were determined. After each egg was individually weighed on an egg scale, groups of eggs were dipped sequentially in each salt solution, starting in the lowest specific gravity solution. The eggs that floated were collected from each solution and assigned the specific gravity of that solution.

Hen Selection

Hens were divided into high, medium and low eggshell quality groups on the basis of their average egg specific gravity. Hens were then selected from the high and low groups on the basis of clutch size. Hens were required to have at least a five day, regular clutch size to be chosen. Blood samples were taken during the fifth, seventh, and ninth months of egg production. The entire selection process was repeated for each sampling period so that different hens were used each time; no hen was used more than once.

Blood Sampling

For each period the selected hens from the high and low eggshell quality groups were each further divided randomly into three sampling times. Blood samples were taken at either 24, 21 or 18 hours post oviposition. This was done so that the blood sample would be taken during the surge of hormone concentration that occurs 2 to 8 hours prior to ovulation.

All hens were kept in the same building, under normal routine, until quietly taken into a separate room for blood sampling. Blood samples (12 ml) were taken by cardiac puncture and .1 ml of .5M oxalic acid was added. Samples were immediately cooled and centrifuged for 20 minutes at 4000 xg. Plasma was decanted and frozen at -20 C until hormones were quantified.

On the day blood samples were taken and on the days before and after sampling, the time of oviposition for each hen was recorded (± 10 minutes). If the hen did not lay at the expected time (i.e., 24 hr ± 1 hr apart), the blood sample was discarded. This was to insure that the

blood sample was actually obtained during the increase in plasma hormone concentrations associated with ovulation.

Progesterone and Estradiol 17β Quantification

Introduction. Plasma progesterone and estradiol were extracted using a double column procedure. The silica gel column procedure was a modification of the technique used by Goldzieher et al. (1961). The Sephadex LH-20 column procedure was an adaptation of the method used by Swanson et al. (1972). Silica gel chromatography separated the lipid and chloresterol fractions from the steroid hormones. Progesterone and estradiol were isolated from other steroids by Sephadex LH-20 chromatography. Following extraction, progesterone was quantified by a double antibody radioimmunoassay modified from Hallford et al. (1975). Estrogen was quantified by a single radioimmunoassay similar to the technique described by Hallford, et al. (in press).

Column Preparation

Silica Gel. Silica gel (100-200 mesh, Fisher Scientific Co.) was washed with methanol 3 to 4 times to remove fines and dried in an oven at 90 C. One half gram of silica gel was weighed into a 16 x 125 mm test tube with 2 ml of hexane and equilibrated overnight.

Columns were 7 mm x 7 cm, tapered to a point, and had a 30 ml reservoir at the top. A small plug of cotton was stuffed into the end of the column. A silica gel slurry was poured into the column and packed gravitationally. A cotton plug was placed at the top of the column. The column was flushed with 10 ml of ethyl acetate followed by 20 ml of hexane.

Sephadex LH-20. Sephadex LH-20 (Sigma Chemical Co.) was equilibrated overnight in chloroform: ethanol (95:5 v:v). Columns (1 x 40 cm) were plugged at the bottom with cotton, above a Teflon stopcock. Sephadex LH-20 slurry was gravitationally packed to a height of 36 cm. A cotton plug was placed at the top of the column bed and a 250 ml reservoir was placed at the top of the column. The column was flushed with 250 ml of chloroform: ethanol.

Extraction

Thawed plasma, 1.5 ml, was vortexed and equilibrated with 6600 dpm of 1, 2, 6, 7 ^{3}H progesterone (105 Ci/mmole, New England Nuclear Co., purified on a Sephadex LH-20 column) and 13000 dpm of 2, 4, 6, 7, 16, 17 ^{3}H estradiol (143 Ci/mmole, New England Nuclear Co., purified on a Sephadex LH-20 column) in a 20 ml screw cap extraction vial for 30 minutes at room temperature. Diethyl ether (10 ml) was added, the extracts were placed on an automatic shaker for 15 minutes, and then frozen overnight. The diethyl ether was decanted into 16 x 125 mm culture tubes and evaporated under N_2 at 40 C. A small amount of benzene (.25 ml) was added to the extract and the extract was layered on the silica gel column. The solvent elution system consisted of 3 ml hexane, 9 ml benzene diethyl ether (95:5 v:v) and 7.5 ml ethyl acetate. Each solvent was added just as the last of the one preceding it entered the column bed. The first 2.5 ml fraction of ethyl acetate that came through the column was collected in a 16 x 125 mm culture tube and evaporated under N_2 at 50 C.

The extract was redissolved in approximately .5 ml of chloroform: ethanol (95:5 v:v) and layered on the Sephadex LH-20 column. Thirty-nine

2 ml fractions were collected in 12 x 75 mm assay tubes. Progesterone was eluted in the same fractions that contained a yellow plasma pigment. Fractions 9 to 11 were combined and dried to 1.5 ml under N_2 at 50 C to obtain the extract used for the assay of progesterone. Estradiol was eluted in fractions 23 to 37. Radioactivity was determined on .5 ml of each fraction in this range. The eight fractions with the greatest radioactivity were combined and dried to 2 ml under N_2 at 50 C to obtain the concentrated extract used for the assay.

To estimate procedural losses of progesterone and estradiol for each plasma sample, the radioactivity of .5 ml of the extract used for the assay procedure was determined. To obtain an estimate of recovery of mass and assay blank values, stripped steer plasma was used. (Stripped plasma is plasma that has been mixed with charcoal and then centrifuged. The steroids will precipitate with the charcoal, resulting in plasma that has very low steroid concentrations.) Two stripped steer plasma samples, stripped steer plasma +5 ng/ml progesterone and stripped steer plasma + 64 mg/ml estradiol were extracted with diethyl ether along with the hen plasma samples, but were not subjected to column chromatography. The steer samples were refrigerated until assayed with the hen samples. These steer samples were used to evaluate the assay system, not the purification technique.

Radioimmunoassay

Estradiol. Aliquots (.3 and .4 ml) of the concentrated fractions from the Sephadex LH-20 columns were pipetted into 12 x 75 mm culture tubes and evaporated under N_2 at 50 C. For each assay of 48 tubes, two standard estradiol curves were prepared by the addition of 0, 1, 2, 4, 8,

16, 32 and 64 pg of estradiol in .1 ml absolute ethanol to culture tubes and evaporated under N_2 at 50 C. Estradiol antisera (.2 ml), #244, provided by Dr. G. D. Niswender, at a dilution of 1:100000 in .05 M phosphate buffered saline and .1% gelatin (PBS + gel, pH 7.0), was added to each assay tube. The solutions were vortexed and incubated for 30 minutes at 4 C. Approximately 50,000 dpm of ^3H estradiol (143 Ci/mmole) in .2 ml buffer was then added to each tube. After vortexing, the tubes were incubated for two hours at 4 C.

Following incubation, the solutions were placed in an ice water bath for 15 minutes. To separate free and bound estradiol, 1 ml of dextron coated charcoal (2.5 g activated neutral Norit and .25 g dextran T-150 per liter of glass distilled water) was added to each tube with all tubes receiving the charcoal solution and being vortexed within two minutes. The solutions were allowed to incubate for eight minutes longer and were centrifuged at 1600 xg at 4 C for 10 minutes. Radioactivity was determined on a .5 ml aliquot of the supernatant diluted with 4.5 ml protein binding counting fluid (21.7 g of 2,5-diphenyloxazole per 3 liters of toluene) in a 10 ml scintillation vial.

Progesterone. Aliquots of 30 and 40 µl of the concentrated fractions from the Sephadex LH-20 column were pipetted into 12 x 75 mm assay tubes. For each assay, two standard curves were prepared by the addition of 0, .025, .05, .1, .2, .3, .4 and .5 mg of progesterone in .1 ml absolute ethanol to assay tubes. The standards and aliquots of plasma extract were evaporated under N_2 at 50 C. Each assay tube received .2 ml of antiprogesterone (Michigan State University 74, diluted 1:3000 in PBS + gel). After vortexing, the solutions were incubated for four hours at 4 C. The solutions were then placed in an ice water bath for 10

minutes. Approximately 10,000 dpm of ^{3}H progesterone (105 Ci/mmole) in .2 ml PBS + gel was added to each tube. The solutions were vortexed and incubated for 12 hours at 4 C. The tubes were placed in an ice water bath and .4 ml of sheep anti-rabbit gamma globulin, at a dilution to give maximum precipitation of the first antibody, was added. The solutions were vortexed and incubated for 48 hours at 4 C. After the final incubation, the solutions were centrifuged at 4 C for 15 minutes. The supernatant (.8 ml) was decanted into 20 ml scintillation vials, diluted with 9.2 ml of protein binding counting fluid and radioactivity quantified.

Statistical Design and Analysis

The data was analyzed as a 2 x 2 x 3 x 3 factorial split-plot incomplete block design. The design was derived in the following way: The plasma extract aliquots for the radioimmunoassay were at two concentrations. There were two eggshell quality groups (high and low) and three sampling times (18, 21 or 24 hours postoviposition). The blood samples were taken during the fifth, seventh and ninth months of egg production (three periods).

The experimental unit was the individual blood sample. Since two aliquots were taken from each sample, a split plot was created in which an aliquot was the subunit and sampling time, eggshell quality group and period made up the main plot. Only 12 tubes (six samples at two concentrations) could be extracted each day. Thus a block was comprised of 12 tubes. Three blocks constituted a replicate in which all 36 treatment combinations were represented once. There were seven replicates. Among the three blocks making up a replicate, sampling time x period was con-

founded in replicate 1 to 4, and sampling time x period squared was confounded in replicates 5 to 7.

The average specific gravity and egg weight of eggs laid by a hen in each period were based on unweighted means. Since egg weights were not recorded in Period 1, the analysis of variance was performed just on data collected from Periods 2 and 3. Least squares equations for estimations of estradiol and progesterone were used to estimate the time before ovulation or after oviposition that the two hormones were maximal.

To obtain approximate estimates of the partial coorelation coefficients, it was assumed that all response variables were obtained by the incomplete block design. The error mean squares and cross products associated with the correlation between estradiol and progesterone that was obtained by this procedure were unbiased. All other estimates of correlation and error are biased. In the analysis for the incomplete block design, the sum of squares and crossproducts for replicates and blocks in replicates were removed from the error. However, since the production traits were not part of the incomplete block design, the sum of squares and crossproducts for replicates and replicates in blocks belong in the error. Therefore, the error mean squares associated with the production traits would be biased downward. The unbiased estimates for these mean squares and crossproducts could be obtained, but it was thought that the cost would not justify the calculations.

The progesterone standard curve was linearized by a log-log transformation and the estradiol standard curve was linearized by a logit-log transformation. A regression equation for each standard curve was then calculated. The concentration of estradiol and progesterone in the samples was calculated from the regression equations of their respective

standard curves and factors to correct for recovery and volume of aliquot were applied to arrive at a per milliliter concentration.

CHAPTER III

RESULTS AND DISCUSSION

For the estradiol assay, the coefficient of variation within an assay was estimated to be 8.3% and the coefficient of variation between assays was estimated to be 20.2%. Between assay variance was controlled by the incomplete block design so that, for this experiment, the overall laboratory coefficient of variation was estimated to be 7.3%. The recovery of mass after the addition of 50 or 100 pg estradiol/ml plasma was 105% (n = 7) and 97% (n = 8). The dose response curves of the samples (n = 11) were parallel to the standard curves ($P < .05$).

The within and between assay coefficients of variation for the progesterone assay were 8.3% and 27.3%, respectively. The incomplete block design controlled the variance between assays so that, for this experiment, the laboratory coefficient of variation was estimated to be 7.0%. The recovery of mass after addition of 2 or 5 ng progesterone/ml plasma was estimated to be 102% (n = 9) and 102% (n = 6). The dose response curves of the samples (n = 8) were parallel to the standard curves ($P < .05$).

The analysis of variance for estradiol and progesterone is shown in Appendix Tables VIII and IX. The average specific gravity, egg weight, egg production, clutch size and hen body weight of the hens in each eggshell quality group for each sampling period are shown in Table II. Specific gravity was a selected trait and, as expected, there is a sig-

TABLE II

AVERAGE SPECIFIC GRAVITY, EGG WEIGHT, EGG PRODUCTION, CLUTCH SIZE, AND HEN BODY WEIGHT OF HENS IN EACH EGGSHELL QUALITY GROUP FOR EACH SAMPLING PERIOD

Period[a]	Eggshell Quality Group[b]	Specific Gravity of Eggs[c]	Egg Weight[c] (g)	Egg Production[d] (Eggs/28d)	Clutch Size[d]	Hen Body Weight[d] (g)
1	H	1.090	----	25.6	16.3	1722
	L	1.084	----	25.2	22.3	1753
2	H	1.090	60.6	25.1	16.0	1756
	L	1.082	60.6	25.0	16.5	1845
3	H	1.089	61.8	23.1	8.1	1783
	L	1.082	60.6	23.9	14.7	1875
S.E.M.		.001	.7	.5	2.5	41

[a]The fifth, seventh and ninth month of egg production.

[b]H = high eggshell quality; L = low eggshell quality.

[c]Based on unweighted means.

[d]Each mean is the average of 21 observations.

nificant difference ($P < .0001$) between the mean specific gravity within each period. The specific gravity of the eggs declined across periods ($P < .05$). This is in full agreement with findings that eggshell quality declines as the hen grows older (O'Neil and Rae, 1952; Mueller et al., 1960; Brenson and Godfrey, 1953). Egg weight and egg production are not different between specific gravity groups. This agrees with Wilhelm (1940) who observed that hens in significantly high or low egg production had no differences between egg weight and shell thickness. However, since it is usually found that increased egg production will result in poorer quality egg shells (Van Tijen, 1977), it would seem that the results from our experiment conflict with this information. But since only hens with good egg production were selected for this experiment, it seems reasonable that egg production would not differ between eggshell quality groups.

The high eggshell quality group had a smaller ($P < .05$) average clutch size than the low eggshell quality group. Although at first it may appear contradictory that egg production is not significantly different between groups while clutch size is, this is possible because clutch size and egg production are measured using different systems. Egg production is the number of eggs laid during a certain time period while clutch size is counted without regards to time boundaries. Thus a hen with a ten day clutch and a two day interval between the end of one clutch and the start of the second will lay 24 eggs in 28 days. A hen with six day clutches, but only a one day interval between clutches, will also lay 24 eggs in 28 days. Clutch size was also used as a basis for hen selection. Thus while clutch size is significantly different between eggshell quality groups, it cannot be determined whether this is

due to the selection process or to some aspect of physiology. It may be possible that because of more frequent pauses in egg production of hens with small clutches, the hen may also be able to replenish her calcium reserves and thus lay eggs with better shells.

Clutch size and egg production declined significantly ($P < .01$) with period, as has been observed previously (O'Neil and Roe, 1952; Godfrey and Jaap, 1949). The hens in the low egg shell quality group were significantly heavier ($P < .05$) than hens in the other group. At this time an explanation for this occurrence cannot be found on the basis of the data from this experiment.

The mean plasma progesterone concentration for all samples in this experiment was 4.75 ± .17 ng/ml and the mean plasma estradiol concentration was 174.41 ± 4.81 pg/ml. These values agree well with other workers that determined plasma estradiol and progesterone concentrations during the surge of hormone associated with ovulation (Peterson and Common, 1972; Lague et al., 1975; Furr et al., 1973; Shahabi et al., 1975; Kappauf and Van Tienhoven, 1972; Shodono et al., 1975).

The plasma concentrations of progesterone and estradiol were not significantly different between eggshell quality groups or between periods (Table III). Both plasma estradiol and progesterone concentrations were significantly different with sampling time (Table IV). Both plasma estradiol and progesterone exhibited a significant quadratic effect ($P < .005$). The concentration of both hormones was higher at 21 hours than at 18 or 24 hours post-oviposition.

Progesterone concentration tended to decline with period (Table V). The basal concentration of progesterone in laying hen plasma has been observed to increase with age (Furr et al., 1973). However, since blood

TABLE III

AVERAGE PLASMA ESTRADIOL AND PROGESTERONE CONCENTRATIONS FOR EACH EGGSHELL QUALITY GROUP DURING EACH PERIOD AVERAGED OVER SAMPLING TIME[a]

Period[b]	Eggshell Quality Group[c]	Estradiol (pg/ml)	Progesterone (ng/ml)
1	H	181.4	5.23
	L	184.3	4.96
2	H	170.8	5.15
	L	160.2	4.48
3	H	181.9	4.09
	L	167.8	4.61
S.E.M.		11.8	.46

[a]Each mean is the average of 42 observations.

[b]The fifth, seventh and ninth month of egg production.

[c]H = high eggshell quality; L = low eggshell quality.

TABLE IV

AVERAGE PLASMA ESTRADIOL AND PROGESTERONE CONCENTRATIONS AT EACH SAMPLING TIME AVERAGED OVER PERIODS AND EGGSHELL QUALITY GROUP[a]

Sampling Time (Hr. Post-Oviposition)	Estradiol (pg/ml)	Progesterone (ng/ml)
18	167.0	2.86
21	194.3	5.83
24	161.9	5.56
S.E.M.	8.3	.29

[a]Each mean is the average of 84 observations.

TABLE V

AVERAGE ESTRADIOL AND PROGESTERONE CONCENTRATIONS IN EACH PERIOD AVERAGED OVER SAMPLING TIME AND EGGSHELL QUALITY GROUP[a]

Period[b]	Estradiol (pg/ml)	Progesterone (ng/ml)
1	182.9	5.09
2	165.5	4.82
3	174.9	4.35
S.E.M.	8.3	.29

[a]Each mean is the average of 84 observations.

[b]The fifth, seventh and ninth month of egg production.

samples for our experiment were taken when progesterone and estradiol would be maximum, not at basal concentration, this data is not readily comparable to that of Furr et al. (1973).

As stated earlier, the blood samples were taken at 18, 21 or 24 hours after oviposition of the first egg of a three day sampling period as illustrated in Figure 1. It was planned that the blood samples would thus be taken during the surge of hormone concentration associated with the ovulation of the egg that would be laid on the third day. However, this assumption is only true if the time period between successive ovipositions (A) is very close to 24 hours. The time interval from the surge of estradiol and progesterone to ovulation of egg 3 (Shodono et al., 1975; Shahabi et al., 1975) and the time interval from oviposition of the second egg to ovulation of egg 3 (Warren and Scott, 1935) vary very little. Thus if time A is longer than 24 hours, the oviposition of egg 2 will move to the right as will the surge of hormone and ovulation of egg 3. Therefore, if a blood sample is taken at 18 hours post-oviposition of egg 1, the sample will no longer be taken during a surge of hormone concentration, but during a time when hormone concentrations are basal.

It is not mechanically possible to wait for oviposition of egg 2 and then take blood samples during the surge of hormone concentration. Instead the samples were taken at 18, 21 and 24 hours post-oviposition and the actual time from sampling to the second oviposition (C) was recorded. Time C gave a better estimate of ovulation time than did time A. The data was analyzed using time C instead of 18, 21 or 24 hours post oviposition. Though the mean square error was reduced, the results of the analysis were much the same; the concentrations of progesterone

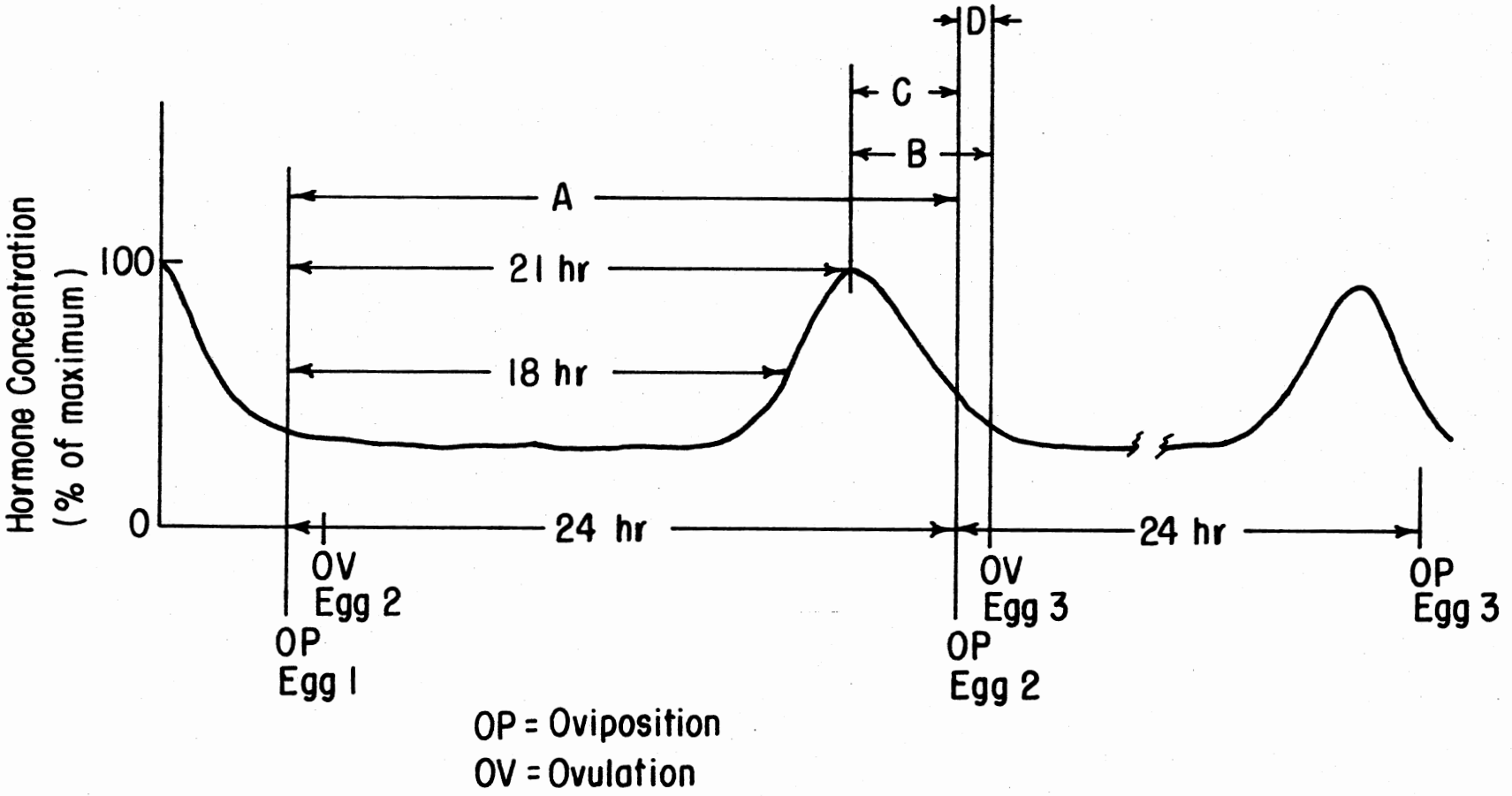

Figure 1. Relationship Among the Times Blood Samples Were Taken, Oviposition, Ovulation, and the Surge of Plasma Estradiol and Progesterone Concentration Associated With Ovulation

and estradiol were only significantly different between sampling times. Estradiol and progesterone had a quadratic effect associated with sampling time. Apparently, the practice of eliminating samples from hens that did not lay egg 2 at 24 ± 1 hour insured, in at least most cases, that the blood sample was taken during a surge of estradiol and progesterone.

Prediction equations were calculated that used both methods of stating sampling time. It was determined that the maximal concentration of estradiol was at 20 hours 53 minutes post-oviposition of egg 1 or 3 hours 49 minutes before oviposition of egg 2. Progesterone was maximal at 22 hours 15 minutes post-oviposition of egg 1 or 2 hours 33 minutes prior to oviposition of egg 2. These maximal values occur at times similar to those described by Peterson and Common (1972), Lague et al. (1975), Furr et al. (1973), Shahabi et al. (1975), Kappauf and Van Tienhoven (1972) and Shodono et al. (1975). This demonstrates that if a hen is laying clutches of seven or more eggs, the eggs in the middle of the clutch will be laid at about 24 hours apart, so that blood samples taken at 18, 21 or 24 hours after oviposition can give a good estimate of the surge of hormone concentration associated with ovulation of the next egg.

The correlation between estradiol and progesterone was .243 ($P < .05$) and the correlation between estradiol and specific gravity was .181 ($P < .10$). Though these coefficients were statistically significant, they are very low, and it is doubtful that they indicate an effect on eggshell quality. All other correlation coefficients were also less than .26 except for the correlation between egg production and clutch size which was .612 and statistically significant at $P < .0001$

(Appendix Table X).

It can be concluded based on these data that plasma progesterone and estradiol concentrations in the laying hen are not related to eggshell quality when measured during the ovulatory surge of hormone. Estrogen can directly affect such aspects of calcium metabolism as plasma calcium and medullary bone formation (Thaeler, 1979), and progesterone and estrogen can indirectly affect calcium metabolism through compounds such as 25 hydroxyvitamin D_3-1-hydroxylase (Tanaka et al., 1978). However, it would appear estradiol and progesterone may not regulate calcium metabolism on a daily basis, and even if they do, are not able to regulate calcium metabolism as related to eggshell quality.

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A P P E N D I X

TABLE VI

RATION COMPOSITION

Ingredients	Percent
Yellow corn, ground	55.21
Soybean meal (44% protein)	15.68
Calcium carbonate	7.24
Tallow (feed grade)	6.52
Meat and bone scrap (50% protein)	5.14
Alfalfa meal (17% protein)	2.63
Live yeast culture[1]	1.68
Distillers solubles	1.68
Dried whey	1.68
Dicalcium phosphate	1.47
Salt	0.50
Vitamin-mineral concentrate	0.50
dl-Methionine	0.07
Calculated analysis:	
Protein, crude % (N x 6.25)	16.10
Metabolizable energy kcal./kg.	2951.
Daily intake (hen/day)	95-100 g.

[1]Manufactured by Diamond V. Mills, Cedar Rapids, Iowa.

TABLE VII

COMPOSITION OF VITAMIN-MINERAL CONCENTRATE

Vitamins and Minerals	Units	Supplies Per kg. of Finished Ration
Vitamin A	U.S.P.	17,637.0
Vitamin D_3	I.C.U.	2,646.0
Vitamin E	I.U.	13.2
Vitamin K	mg.	6.6
Vitamin B_{12}	mg.	0.018
Riboflavin	mg.	8.8
Niacin	mg.	70.4
Pantothenic Acid	mg.	17.6
Choline Chloride	mg.	1,102.0
Manganese	mg.	60.9
Iodine	mg.	1.9
Cobalt	mg.	1.3
Iron	mg.	48.1
Copper	mg.	3.6
Zinc	mg.	50.0

TABLE VIII

ANALYSIS OF VARIANCE FOR ESTRADIOL

	df	MS
Main Plot		
Replicate	6	7,983
Block	14	15,277
Sampling time	2	25,559
linear	1	1,054
quadratic	1	50,064
Period	2	6,344
Sampling time x period	4	4,507
Eggshell quality group	1	3,308
Sampling time x eggshell quality group	2	983
Period x eggshell quality group	2	1,678
Sampling time x period x eggshell quality group	4	1,655
Error	88	5,829
Subplot		
Aliquot size	1	4,479
Sampling time x aliquot size	2	1
Period x aliquot size	2	6
Sampling time x period x aliquot size	4	55
Eggshell quality group x aliquot size	1	354
Sampling time x eggshell quality group x aliquot size	2	119
Period x eggshell quality group x aliquot size	2	427
Sampling time x period x eggshell quality group x aliquot size	4	154
Error	108	162
Total	251	3,570

TABLE IX

ANALYSIS OF VARIANCE FOR PROGESTERONE

	df	MS
Main Plot		
Replicate	6	9.4
Block	14	7.1
Sampling time	2	225.6
Linear	1	303.9
Quadratic	1	147.3
Period	2	11.9
Sampling time x period	4	2.8
Eggshell quality group	1	1.2
Sampling time x eggshell quality group	2	5.7
Period x eggshell quality group	2	7.7
Sampling time x period x eggshell quality group	4	10.7
Error	88	7.3
Subplot		
Aliquot size	1	2.7
Sampling time x aliquot size	2	0
Period x aliquot size	2	.4
Sampling time x period x aliquot size	4	.6
Eggshell quality group x aliquot size	1	0
Sampling time x eggshell quality group x aliquot size	2	0
Period x eggshell quality group x aliquot size	2	.1
Sampling time x period x eggshell quality group x aliquot size	4	.1
Error	108	.1
Total	251	5.4

TABLE X

CORRELATION COEFFICIENTS

	Progesterone	Eggshell Quality Group	Egg Production	Clutch Size	Body Weight
Estradiol	.243**	.181*	.012	.033	.140
Progesterone		.116	.087	-.117	.016
Eggshell quality group			-.259	-.054	-.164
Egg production				.612**	.029
Clutch size					.209**

*P < .01.

**P < .05.

VITA[2]

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