STUDIES ON THE CAUSE AND INCIDENCE OF BLOOD SPOTS IN CHICKEN EGGS

By

PHILIP GLENN STILES

AN ABSTRACT

Submitted to the School for Advanced Graduate Studies of
Michigan State University of Agriculture and
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ABSTRACT

The presence of a blood or meat spot in an egg causes that egg to be classed as C quality if it is less than one-eighth of an inch in diameter and as inedible if it is over one-eighth of an inch in diameter and is, therefore, an economic loss. This study approached the problem from a physiological and environmental viewpoint by attempting to add evidence as to the cause and influencing factors in the incidence of blood spots.

A technique was developed whereby the red blood cells of a laying hen were labeled with radioactive phosphorus (P^{32}) and could be traced into a blood spot. Blood spots formed after the P^{32} labeled cells were injected had detectable radioactivity when the blood spots contained more than 0.024 milliliter of blood. This technique may be used to determine the total blood volume in a hen and in a blood spot by comparing their counts per minute per unit of volume with the counts per minute of the volume injected. More important, however, the method can be used to determine the time a blood spot forms in hens with known ovulatory cycles. A hen injected 9 hours prior to ovulation had a radioactive blood spot, while another hen injected 3 hours after ovulation had a non-active blood spot.

In comparing normal and blood spot eggs of a similar clutch position, it was found that the mean oviposition time of eggs containing blood spots occurred approximately 30 to 50 minutes

sooner than normal eggs. A higher incidence of blood spots was found in the first egg of a clutch than in eggs of succeeding clutch positions. No correlation was found between the barometric pressure or net changes in pressure for the area and the incidence of blood spots in eggs laid 24 or 48 hours later. The mean weight of eggs containing blood spots was found to be significantly more than the mean weight of normal eggs in similar clutch positions. The average weight of the blood spot eggs ranged from 0.77 to 1.93 grams heavier than normal eggs.

Physical disturbance treatments did not substantially affect egg production, blood spot incidence or other interior quality factors. Scaring birds 3 times a day decreased shell thickness by 0.00031 of an inch. High and low frequency sound treatments also had little effect on egg quality or blood spot incidence. Continuous light and short intermittent light periods increased blood spot incidence, albumen height, egg weight and Haugh score over the normal 14 hours of light followed by 10 hours of darkness.

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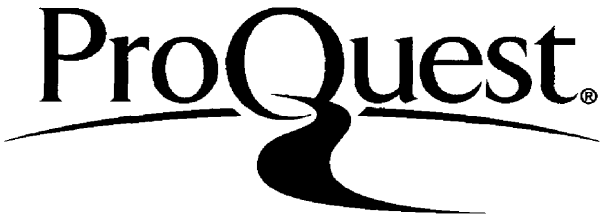

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INTRODUCTION

The problem of blood spots in chicken eggs has existed since the beginning of commercial egg production and currently causes from 2 to 4 percent of the eggs produced to be classed as loss. This problem has been studied from many different aspects and much information on the subject can be found in the literature; however, none of the research work has brought about the elimination of blood spots and only a few of the studies have shown that the incidence could be controlled to a limited extent.

Blood spots in eggs are not a public health hazard and are classified as inedible or loss purely for psychological reasons. A bright red blood spot showing on a lustrous yellow yolk does not have an appetizing appearance; therefore, the modern housewife refuses to eat it and consequently may not make further egg purchases from the same source. The economic losses due to not purchasing eggs after finding one or more blood spots can not be accurately measured, but are undoubtedly of major importance.

In this study the blood spot problem was investigated from the physiological viewpoint by utilizing radioactive isotope tracers with ovulatory cycle analysis and also from the standpoint of the influence of specific environmental elements. The first experiment consisted of developing a procedure for labeling the red blood cells in a laying hen in such a way that blood spots

in an egg from this hen would show radioactivity in detectable amounts. Thus, by labeling the blood at a specific time in the ovulatory cycle one could determine when a blood spot was formed.

Another objective, studied in the second experiment, was to determine if ovulation time was different for eggs containing blood spots than for normal eggs. In addition, this experiment was designed to discover if there was a relationship between the incidence of blood spots and egg clutch position, barometric pressure, egg weight, and egg albumen height.

A third objective of this research was to investigate the influence of physical disturbance, sound, and light variations on the incidence of blood spots and other egg quality characteristics. Each of the environmental conditions were studied separately to determine their individual effects.

REVIEW OF LITERATURE

Incidence and Economic Loss

The occurrence of blood spots in chicken eggs has been known since early history. According to Romanoff and Romanoff (1949), Aristotle believed blood spots resulted from premature yolk expulsion. In 1600 Fabricus thought the presence of blood spots was evidence that egg yolks are formed directly from blood. Many concentrated studies of blood and meat spots have been made since 1930.

There is a considerable economic loss due to blood spots. Dawson (1953 and 1955) reported a $700,000 annual loss to Michigan producers in 1953 and over one million dollars in 1955 due to removing blood spot eggs after candling. The total economic loss is undoubtedly much greater than these figures since the small blood spots that are not detected by the candlers may cause a lower consumer demand and consequently fewer eggs are purchased and consumed. One Michigan egg buying station had a candling loss of $1\frac{1}{2}$ percent of all its eggs due to blood and meat spots. In eggs broken out from 2,438 New Hampshire hens, Dawson and Richardson (1952) found 64.8 percent of the eggs contained spots. Of these 48 percent were on the yolk, 27 percent were free floating in the albumen, and 25 percent were in the chalaza. Twenty percent of the eggs with spots had them in more than one position.

Five percent of the yolk spots were white while 50 percent were red; however, in the albumen 50 percent were white, 2 percent were red, and the remainder were a shade of brown. Most of the spots in the chalaza were brown. Miller et al. (1957) reported eggs selected from a grading station prior to candling contained over 3 percent blood spots with 0.96 percent being over one-eighth of an inch in diameter. Most blood spots were one-thirty-second of an inch or less in diameter.

Nalbandov and Card (1947) reported the incidence of blood spot eggs from 10 midwestern Leghorn flocks averaged 12.5 percent. Of this 7.7 percent were under one-eighth of an inch in diameter. Meat spots were found in 12.8 percent of the eggs with 10.2 percent being under one-eighth of an inch in diameter. The sample of eggs from heavy breeds contained 41.7 percent blood and meat spots of which 11.4 percent were over one-eighth of an inch in diameter.

Merritt (1950) reported 20 percent of the turkey eggs examined contained either blood or meat spots, mostly white meat spots. White meat spots were found in 31 percent of the duck eggs broken out and 19 percent of the pheasant eggs contained meat spots.

Another possible economic loss may result from the egg being more susceptible to bacterial spoilage when it contains a blood spot due to the more easily assimilable nutrient material available for bacteria (Romanoff and Romanoff, 1949).

Origin

Nalbandov and Card (1944) reported that bleeding may occur at ovulation and cause blood spots, but this happens only in rare instances. More frequently blood spots are caused by intra-follicular bleeding, which may have occurred several days before ovulation. In observations of laparotomized hens, they found yolks released with both large and small blood clots adhering to them although no hemorrhage had taken place at ovulation. Hemorrhages may take place anywhere in the follicle. Sheet-like or narrow, elongated masses of blood on the yolk are caused by large masses of blood diffusing between the yolk and follicle. A severe hemorrhage can cause a wide sheet of blood to cover most of the yolk surface, or may accumulate in the hollow follicle stalk becoming conical or globular in shape and may eventually be free floating in the albumen. Sub-membranous clots are most commonly found attached to the vitelline membrane and remain there throughout ovulation.

For many years meat spots were reported as being degenerate blood spots caused by normal changes in pH or temperature or both, and that the presence of a meat spot indicated the bleeding took place several days before ovulation, while blood spots were caused by bleeding immediately prior to ovulation (Nalbandov and Card, 1947). Nalbandov and Card, (1944) found that artificial blood clots were changed into meat spots after 10 days at 88 degrees

Fahrenheit and a pH of 8.0 to 9.2. Exposure to air and unsterile technique hastened the transformation of blood clots to meat spots.

Merritt (1950) reported high temperatures (40 degrees centigrade) caused blood clots to become lighter in color, whereas refrigeration temperatures (4 degrees centigrade) did not. His work indicated that any degeneration of blood spots into meat spots after oviposition was insignificant. Meat spots were much more prevalent in eggs from heavy breeds than in eggs from light breeds of chickens. Merritt stated that it was conceivable that a large percentage of the white meat spots resulted from accumulations of oviducal excretions, which is in partial agreement with Lucas (1946) but in disagreement with Nalbandov and Card (1944), who stated white meat spots resulted from blood clot degeneration. Attempts to induce inclusions in eggs through operative and insertion techniques in this study were unsuccessful.

Burmester and Card (1938) made a microscopic examination of meat spots which showed that dark red meat spots consisted of large masses of red blood corpuscles surrounded by a thin layer of protein. Very little degeneration of red blood cells took place in the dark red meat spots. Some initial degeneration changes had taken place in the brownish colored meat spots. There was a wide variation in the microscopic structure of meat spots ranging from those with a large amount of non-cellular proteins and very few red cells to those showing no evidence of

cells in any phase of degeneration. All but 3 percent of the meat spots examined had evidence of having contained red blood cells. There were no normal or abnormal cellular tissues other than blood found in any meat spot except for a few bacteria found in a small percentage of the meat spots.

Helbacka and Swanson (1958) formed the opinion that colored meat spots found in white shelled eggs are normally degenerated blood, but those found in brown shelled eggs appeared to be of two kinds: (1) those having their origin in degenerated blood and being non-fluorescent under ultra-violet light; or (2) those containing a considerable quantity of calcium and being fluorescent because of their porphyrin content. Protoporphyrin is the pigment in colored meat spots that give the fluorescent property. They concluded that the majority of meat spots in shell eggs do not have their origin in degenerated blood.

Nutritional Effects

The incidence of blood spots is influenced to some extent by nutritional factors. Nalbandov and Card (1947) reported a large decrease in the number of blood spots in the eggs from hens allowed to range for 3 or 4 weeks. They assumed that fresh green grass could prevent follicular hemorrhages. In disagreement with this, Denton (1947) indicated that the ingestion of green grass and range had no appreciable effect on the incidence of blood and

meat spots. He also found that vitamin K had no effect on their incidence.

Carver and Henderson (1948) reported that the addition of rutin and ascorbic acid to the ration did not reduce the incidence of blood spots.

Sauter, Stadelman and Carver (1952) found that hens fed a ration containing 10 percent alfalfa consistently produced a lower incidence of both large and small blood spots than did hens fed a ration containing no alfalfa. The Leghorns on the alfalfa ration averaged 0.6 percent lower blood spot incidence and New Hampshires averaged 5.9 percent lower incidence.

Bearse, McClary and Saxena (1953) reported that hens receiving low levels of vitamin A (200 International units per 100 grams feed) showed a significantly higher blood spot incidence than hens fed higher vitamin A levels. Bearse (1955) found that a decrease in vitamin A below 2000 International units per pound caused an increase in the blood spot incidence. Levels of vitamin A above 1700 United States Pharmacopeia units caused the maximum benefit in this study. Hens fed rations containing only 908 International units of vitamin A consistently laid eggs of a higher blood spot incidence than did similar hens fed 2000 International units of vitamin A. Vitamin B_{12} supplementation appeared to have some effect in lowering the blood spot incidence.

Bearse (1958) reported that birds which had received

additional levels of vitamins D, E and K separately showed no difference in blood spot incidence; however, the addition of excess vitamins A, D, E and K together decreased blood spots slightly. Birds fed 6000 International units of vitamin A per pound had fewer blood spots than did birds fed 3000 International units of vitamin A. Increasing the energy level from 1115 to 1450 Calories per pound resulted in an increase in blood spots.

Genetic Effects

More has been accomplished toward reducing the incidence of blood spots by a systematic genetic selection of breeding stock than by any other means. Quinn and Godfrey (1940) reported there were significant breed and family differences in the blood spots occurring in both the yolk and the albumen. They found no significant correlation between percentage of blood spots and egg production, egg weight, or body weight.

Jeffrey (1945) reported marked breed differences existing in the percentage of eggs with red meat spots. Brown eggs produced by heavy breeds contained between 12.6 and 20.3 percent red meat spots, whereas Single Comb White Leghorn eggs had as low as zero percent red meat spots. Similar breed differences were found for pale meat spots.

Lerner and Taylor (1947) reported no significant reduction in blood spot incidence when blood spot eggs were removed before

the eggs were set in the incubators during the hatching season. By selecting for a high and low incidence of blood spots in a strain of Rhode Island Reds which had an original average of 61.9 percent blood spots, Quinn et al. (1948) developed two separate strains after six years of selection. One strain averaged 80 percent blood spots and the other 20 percent. Environment and season appeared to have little influence on blood spot incidence in this study.

Quinn and Lee (1949) crossed Rhode Island Reds with White Leghorns and found no apparent relationship between shell color and the percentage of blood spots in eggs from the F_1 generation of this cross. Jeffrey and Walker (1950) reported there is an independence in the incidence of blood spots and meat spots and that separate genes probably control their production. Their evidence showed that dark or medium brown eggs contained more colored meat spots and fewer white meat spots than lighter colored eggs.

Stadelman et al. (1952) reported there was no relationship between blood spot incidence and shell color or between the incidence of blood and meat spots. There was a significant relationship found between shell color and the color of meat spots.

Lerner, Taylor and Lowry (1951), by ordinary commercial candling, detected blood spots in 0.5 to 1.33 percent of the eggs from a production bred flock of Single Comb White Leghorns. This

percentage increased to 23 percent by selection for a high incidence. Dawson and Richardson (1952) reported Leghorns had a lower incidence of blood and meat spots than did the heavy breeds on a broken out basis. A seasonal variation and a strain variation were reported for both Single Comb White Leghorns and heavy breeds.

Dawson et al. (1954) found considerable variation between strains and individuals for albumen firmness, shell thickness, and the incidence of blood and meat spots. McClary and Bearse (1954) selected for above average shell thickness among several Single Comb White Leghorns and found they had a higher blood spot incidence than strains not selected for shell thickness. A positive genetic correlation of 0.44 was calculated for egg shell thickness and percentage of blood spots.

Farnsworth and Nordskog (1955) estimated the heritability of blood spots to be 0.32 for approximately 15 eggs candled per hen. No significant correlations were found when blood spots were compared with egg production, sexual maturity, or egg weight.

Physiological Influence

Lucas (1946) made smears from blood spots from fresh eggs and found great variability in the relative cell type frequencies. The range of lymphocytes was from 3 to 60 percent, eosinophils from 8 to 74 percent, basophils from 0 to 19 percent, and macrophages from 4 to 85 percent. Differential blood counts averaged

38.3 percent lymphocytes and monocytes, 52.3 percent eosinophils (all types) and 10.5 percent basophils. The lymphocyte-monocyte group appeared more susceptible to damage than the other two. The evidence in this study indicated that the connective tissue cells and cells of extravascular origin were carried into the egg.

Helbacka and Swanson (1958) reported that colored meat spots have a fluorescent property similar to that of egg shell pigments when exposed to ultraviolet light. Neither blood spots nor degenerated blood possess this quality. Significant correlations were found between egg shell color and meat spot color, meat spot color and meat spot fluorescence, and egg shell color and meat spot fluorescence. The oviducts of autopsied hens contained tissue similar to meat spots in eggs. These tissues were concentrated mainly in the uterus. Hens with a high incidence of meat spots in their eggs usually had a high incidence of abnormalities of the oviduct.

Age appears to have some effect on blood spot incidence. Nalbandov and Card (1944) reported 44 percent of all hens sampled laid eggs containing blood spots their first year. The blood spot incidence decreased in the second laying year for 60 percent of the hens and increased for 35 percent. Jeffrey (1945) reported the size of pale and red meat spots tended to decrease as the laying year progressed. Lerner (1946) found that birds not surviving their first laying year had a lower blood spot incidence than those that lived into their second laying year. The difference

was not significant.

There is a gradual decrease in egg weight with each succeeding egg in a clutch, and the longer the clutch the greater is the decrease from the first to the last egg, but the smaller is the decrease of each egg within the clutch (Bennion and Warren, 1933). Since all of the eggs of a clutch are not equal in size it is logical to assume that this could have an influence on blood or meat spots. The relationship between size and color of meat spots to clutch position was determined by Jeffrey (1945). Tiny pale meat spots reached a peak of 32.1 percent at the number 5 clutch position, then declined to 20.8 percent at the sixth clutch position. Another increase followed until a high peak of 41.2 percent was reached. The percentage of large red meat spots was highest in eggs of clutch position 1 (14.9 percent) followed by a sharp decline until a second peak was reached at positions 6, 7 and 8. No large red meat spots were found in eggs laid after the 9th clutch position. There was no significant difference in the incidence of meat spots previous to and following the pause in egg production. The first egg laid following a pause constituted a very significant change in both egg shell color and meat spot color. Sauter et al. (1952) reported little correlation between the number of pauses (14 days or more) and the overall percentage of blood spots. Intensity and persistency of lay were reported to have little effect on blood spots.

Stadelman and Sauter (1952) reported that hens that produce

12 consecutive blood spot free eggs lay less than 20 percent blood spots with 99 percent reliability and less than 15 percent with 95 percent reliability. Thirty-six consecutive clear eggs indicate hens lay less than 5 percent blood spots with 99 percent reliability.

Strain and Johnson (1957) found eggs in the first clutch position had the highest incidence of blood spots (12.9 percent) and the second clutch position had the second highest incidence (10.1 percent) of blood spots.

Bastian and Zarrow (1955) developed a hypothesis for the asynchronous ovulatory cycle of laying hens. They reported ovulation is not dependent on or caused by oviposition, but more by the release of luteinizing hormone from the pituitary 6 to 8 hours before ovulation. This becomes later with each egg in the clutch and thus causes each succeeding egg to be ovulated later. They believed that 2 separate and independent cycles interact in such a way as to result in the typical ovulatory cycle of the hen. These 2 cycles are: (1) the 24 hour day-night rhythm; and (2) the rhythmic maturation of ovarian follicles. The implications drawn from these two cycles are that each succeeding ovum in a clutch is ovulated at a progressively earlier state of development and is therefore smaller. Succeeding ova within a clutch, being less mature, should require progressively higher levels of luteinizing hormone to ovulate them at equal periods prior to their expected ovulation.

Environmental Influences

The environment within which a hen is placed certainly has a major influence on both egg production and egg quality. The influence of the environment on the incidence of blood spots has been divided into the effects of season, light and activity, sound, and irradiation.

Significant seasonal effects on blood spot incidence have been noted by Lerner and Smith (1942), Jeffrey (1945), Lerner and Taylor (1947), Sauter et al. (1952), Dawson (1955) and Strain and Johnson (1957). Seasonal influences were reported to be non-significant by Denton (1947) and King and Hall (1955). Jeffrey (1945) found (from December until the following August) a marked seasonal decline in the incidence of all sizes of blood spots for all breeds of chickens examined except the Barred Plymouth Rocks. Blood spot incidence in eggs from Barred Plymouth Rocks increased until March and then declined. Lerner and Taylor (1947) found a seasonal increase in blood spot incidence beginning with production in the pullet year and reaching a peak in June. Sauter et al. (1952) reported that the lowest blood spot incidence occurred in mid December and high peaks in February, March and July. Dawson (1955) found the highest blood spot incidence in February, March and April. Strain and Johnson (1957) found the lowest incidence in October.

Many workers have studied the effect of light on egg quality

and production. Penquite and Thompson (1933) reported that continuous lights did not significantly change the total number of eggs laid but did cause more eggs to be laid in the winter. Parkhurst (1933) found no significant difference in the egg weight of White Leghorn pullets in lighted pens and unlighted pens. Mueller et al. (1951) reported that the effects of light on egg quality and blood spot incidence were inconsistent. Egg quality was highest under controlled light and temperature conditions. Wilson and Abplanalp (1956) reported that intermittent light at regular cycles of 4 hours gave higher egg production than the same amount of continuous lighting. Egg production obtained under short photoperiods was not proportionate to the amount of light given. The minimum amount of light needed for an all-or-none response for maintaining egg production in pullets is probably less than 6 evenly spaced one-minute photoperiods in 24 hours. Hens were more susceptible than pullets to light fluctuations, and good layers were more resistant to changes than poor layers. Lanson and Sturkie (1958) reported the time of oviposition could be altered as much as 6 hours by the manner of placement of one-half hour light periods. When the amount of darkness was reduced to 2.5 hours per day, eggs were laid an average of 14 hours after the beginning of the dark period. Pullets receiving 10 hours of darkness laid eggs an average of 16 hours after the start of the dark period. Darkness did not have an accumulative effect in controlling the time of

oviposition when one-half hours of darkness were separated by one-half hours of light. Ostmann and Biellier (1958) found that hens put on rhythmic light-dark "days" of less than 24 hours in length decreased their average clutch length and hen-day egg production. Increasing periods of day-length progressively advanced the time of oviposition to an earlier hour of the day.

Jeffrey and Pino (1943) frightened laying hens with barking dogs and found that this treatment did not increase the incidence of blood spots in eggs. They also reported that eggs from birds kept in cages had a lower blood spot incidence than did eggs from birds on the floor. In contrast to this McDowell (1958) reported that eggs from caged birds had more blood spots than eggs from floor birds.

Van Went (1954) tested the effects of ultrasonic and ultrashort waves on humans. He found the human body parts influenced in a mechanical way by ultrasonic waves suffered from treatment with low frequencies rather than high frequencies. Ultrasonic waves caused hemolysis and ultrashort waves applied directly to the body caused vasodilation.

Smith et al. (1956) reported no increase in blood spotting by oviduct irradiation. The ovary was shielded from the radiation and showed no evidence of radiation damage. After irradiation the albumen contained an opaque material giving the albumen a milky appearance. The egg shells from treated hens often had abnormal shapes.

Detection of Blood Spots

At warm temperatures the albumen becomes more watery with age and allows the yolk to come closer to the surface enabling the candler to detect more blood spots. Jensen et al. (1952) reported that a larger percentage of blood spots present can be detected by candling eggs after they are several days old than when they are first laid. Approximately 40 percent of the blood spots degenerated or disappeared when eggs were stored several weeks. Blood spots less than one-eighth inch in diameter disappeared in larger percentages than those over one-eighth of an inch.

Brant et al. (1953) reported that eggs containing blood spots over one-eighth of an inch in size consistently absorbed lengths of light near 575 millimicrons. An instrument was developed that automatically scanned eggs with light waves between 585 and 565 millimicrons. Eggs containing no blood could be segregated with 99.7 percent accuracy. Eggs containing blood spots over one-eighth of an inch in diameter were segregated with 98.1 percent accuracy, while those with blood spots less than one-eighth of an inch in diameter were identified with only 28.6 percent accuracy. Hood (1956) reported that an electronic machine manufactured by Librascope, Incorporated, could automatically detect and segregate blood spot eggs at the rate of 7,200 eggs per hour.

Radioactive Phosphorus

There are many reports in the literature regarding the use of radioactive phosphorus with chickens. Practically all of these reports involved feeding P^{32} or injecting it intramuscularly and then recording the phosphorus uptake in the various tissues and components of the egg.

Feeding.-- Shirley et al. (1951) reported that following an oral administration of P^{32}, the dose that was excreted in the excrement during the first, second, third, and fourth days was approximately 35, 7, 1, and less than 1 percent respectively. After the tenth day the values leveled off within the range of 0.0001 to 0.001 percent. No P^{32} isotope could be detected in the excrement after 65 days. After 130 days P^{32} could be detected in several of the bones, but not in the muscle, nerve tissue, blood or eggs.

Clegg et al. (1954) fed radioactive phosphorus to hens to determine the distribution of phosphorus in each of the electrophoretic components. They developed an extraction procedure for preparing a clear solution containing all of the yolk proteins.

Patrick and Schweitzer (1952) found that vitamin D is required for phosphorus absorption from the digestive tract.

Subcutaneous and Intramuscular Injections.-- Shirley et al. (1952) using radioactive phosphorus and calcium found that the

duodenum, small intestine, ceca, and cloaca are the principle sites of calcium and phosphorus excretion. Very little was excreted in the large intestine. These same workers in 1954 determined the rate of deposition and turnover of the isotopes in 18 principle tissues at intervals between 0.25 hours and 21 days after intramuscular injections of P^{32}. During the same year these workers reported that two hens receiving approximately 20 millicuries of P^{32} intramuscularly over a period of 7 months died within 8 months of the initial administration. Each hen had lost approximately 200 grams of weight but showed no reduced vitality until death. However, they showed great numbers of petechial hemorrhages throughout the heart, ovaries, and magnum, a necrotic right lobe of the liver, an abnormal green substance in the small intestinal tract, and blood in the ceca. Approximately 43 percent of the P^{32} injected into the hens was excreted in the excrement within 10 days after administration. In general, the hen is very resistant to radiation damage.

Uptake of P^{32} in the Egg.-- Lorenz, Perlman, and Chaikoff (1943) reported that when P^{32} was injected subcutaneously, shells from eggs laid within one hour showed radioactivity. Activity was greatest in those shells that were being formed at the time of injection. No appreciable activity was found in the albumen of eggs for 24 hours. Radioactivity reached a maximum in 24 to 54 hours and then steadily declined. Yolks showed only traces

of P^{32} during the first 24 hours following injection. Maximum yolk activity was found in the period between 98 to 146.8 hours after injection. O'Neil (1948) substantiated this evidence when he found a maximum activity in the shell within 24 hours, 48 to 72 hours for the white, and 144 hours for the yolk.

Randles (1954) reported most of the phosphorus in the fresh egg is found in the yolk as phospholipid complexes. In the developing embryo these lipids become dissociated and the free phosphate is mobilized to the living organism. By using an allantoic injection of a phosphate buffer labeled with phosphorus, he found very little or none of the tracer diffused into the yolk sac; and concluded that there is only a unidirectional diffusion of phosphate out of the yolk sac.

Smith, Winget, and Blackard (1954) reported that upon injecting P^{32} intramuscularly less than 2 percent of the dose was transferred to the shell and less than 1 percent was transferred to the white in 12 days, while 10.8 percent was transferred to the yolk in the same period of time. In this study he used the following formulas.

$$\text{Specific Activity} = \frac{\text{mc } P^{32}}{\text{mgms. phosphorus}}$$

$$\text{Organic Specific Act.} = \frac{\text{Total activity - inorganic activity}}{\text{Total phosphorus - inorganic phosphorus}}$$

$$\text{Standard Specific Act.} = \frac{\text{mc } P^{32} \text{ / mgm phosphorus}}{\text{mc } P^{32} \text{ injected / kg body wt.}}$$

Effect of Radiation on Blood.-- Dixon (1948) gave lethal and sublethal doses of P^{32} to 17 day old chicks and observed the red blood cells and various leucocytes until death, or at a time of sacrifice 17 days later. He found virtual agranulocytosis after 7 days of intense P^{32} radiations. There was a marked decrease of total red and white cells. In contrast to this Shirley et al. (1954) reported P^{32} had no observed effect on the total red and white cell counts, however, no monocytes or neutrophiles were observed for three weeks prior to death.

Techniques of Labeling Human Blood Cells with P^{32}.-- Beierwalters, Johnson, and Solari (1957) reported a technique for blood volume determinations in humans using radioactive phosphorus. Their technique was as follows: Draw 15 milliliters of blood into a heparinized syringe. Place 5 milliliters of blood into a sterile, rubber-capped, centrifuge tube, and incubate at 37 degrees centigrade with constant rotation with 0.5 millicurie of radioactive isotonic dihydrogen phosphate with pH of 7.3. At the end of 2 hours the red cells are washed 3 times by adding isotonic saline solution, centrifuging, and removing the supernatant fluid. After the third washing with saline, some of the plasma obtained from the remainder of the patient's blood and containing no P^{32}, is added to the cells to reconstitute whole blood with a hematocrit of approximately 40. One milliliter of this blood is then injected into an antecubital vein, and using the same needle and

syringe 1 milliliter is placed in a 2000 milliliter flask and water is added to the 2 liter mark (Standard). Fifteen minutes after injection, 5.0 milliliters of blood is drawn from the antecubital vein in the opposite arm and placed in a dry oxalate tube. Then 0.1 milliliter of this blood sample, and the standard, are each pipetted into aluminum planchets in triplicate and counted in a pig under a Geiger-Mueller tube.

$$\text{Total Red Cell Vol.} = \frac{\text{CPM / 0.1 ml. Standard X 2,000 X hematocrit}}{\text{CPM / 0.1 ml. blood}}$$

$$\text{Blood Volume} = \frac{\text{Total red cell volume}}{\text{Hematocrit}}$$

EXPERIMENT I. LABELING BLOOD SPOTS IN CHICKEN EGGS WITH RADIOACTIVE PHOSPHORUS

The objective of this experiment was to develop a procedure for labeling the red blood cells in a laying hen in such a way that blood spots in an egg from this hen would show radioactivity in detectable amounts.

Radioactive phosphorus (P^{32}) decays into sulfur (S^{32}) by emission of a beta particle. The beta particle is a negatively charged electron arising from a neutron in the nucleus of the atom. This reaction can be shown as:

$$_{15}P^{32} \longrightarrow {}_{16}S^{32} + {}_{-1}B^{0}$$

Radioactive phosphorus has a half life of 14.3 days. Since red blood cells absorb phosphorus-32 when incubated with radioactive isotonic sodium dihydrogen phosphate, such labeled cells can be traced throughout the body of the hen. If the labeled cells are injected into a bird at known times of the ovulatory cycle, the time of subsequent blood spot formation can be correlated to this cycle. Beirwalters, Johnson and Solari (1957) reported a technique for blood volume determinations in humans using radioactive phosphorus. A modification of their technique was followed in developing this procedure for labeling blood spots in eggs.

Experimental Procedure

Four Single Comb White Leghorn hens with a known high blood

spot incidence were kept in separate cages and checked hourly during the day to determine their exact ovulatory cycle. Due to a lack in sufficient quantities of P^{32} only one hen was used at a time in developing this technique. A known volume of radioactive isotonic sodium dihydrogen phosphate ($Na\ H_2\ PO_4$) was neutralized to a pH of 7.3 by the addition of sodium hydroxide. A one lambda sample (0.001 milliliter) was taken to determine the amount of radioactivity exposed to the blood cells. Four and one-half hours before the hen was due to ovulate, a 5 milliliter blood sample was withdrawn from the brachial vein in a heparinized syringe. The radioactive isotonic dihydrogen phosphate was carefully added to the 5 milliliters of whole blood and thoroughly mixed with bubbling air. The mixture was immediately divided into four equal amounts in four Warburg flasks and placed in a Warburg water bath to incubate. The Warburg water bath maintained a constant temperature at 39 degrees centigrade and kept the flasks in a continuous shaking movement. The optimum incubation time was approximately three hours. During this period the flasks were allowed plenty of fresh air to prevent the cells from becoming deoxygenated.

After the mixture of blood and P^{32} had completed the incubation period, the cells were separated by centrifugation at 3000 revolutions per minute for 12 minutes. The volumes of both supernatent and blood cells were noted. Three 0.01 milliliter

samples of supernatant were tested for radioactivity. Physiological saline was added to the cells to bring the total volume to 5 milliliters. This mixture was thoroughly blended by carefully bubbling air through it, thus minimizing the mechanical damage to the cells caused by using a stirring rod. A glass stirring rod was used to mix the P^{32} with the cells in the first two preliminary trials, but bubbling air was used in later trials. The cells were then washed by centrifuging at 3000 revolutions per minute for 10 minutes, after which the supernatant was again removed and three samples of 0.2 milliliters were taken. This washing process was repeated three times to insure the removal of all excess P^{32} from the surface of the blood cell.

The labeled cells were resuspended in physiological saline in the exact hematocrit proportion and were injected into the brachial vein at a known stage of the ovulatory cycle. Samples for radioactivity and blood volume measurement were taken of the blood injected and also of the circulating blood at various times after injection. Fecal samples were taken hourly for the first few hours after injection and daily thereafter. All of the samples were dried for 24 hours at room temperature before they were counted.

Results and Discussion

The whole blood had strong radioactivity within 10 minutes after injection while the plasma registered no activity for 45

hours. This indicated that all of the P^{32} was contained within the blood cells and did not escape until normal metabolic transfer shifted some of it into the plasma and tissues. The disappearance of P^{32} from the cells was rapid as illustrated in Figure 1. Consequently, this procedure gives radioactivity which is confined only to the blood cells within the blood spot if the cells are injected within a few hours prior to blood spot formation. If the time between injection and blood spot formation is increased to more than 24 hours, the yolk also becomes radioactive. By knowing the exact ovulatory cycle of the hen and by timing the P^{32} injection to fit into this cycle, the exact time of blood spot formation can be determined. Due to the low percentage of eggs that show sizable blood spots, several hens should be injected at one time to carry out the experiment properly.

Hen 228 was injected approximately 3.5 hours after the predicted ovulation time of an egg containing a large blood spot. The blood spot was not radioactive showing that this hen, whose normal time between ovulation and oviposition was 28 hours, could not have formed the blood spot between the time of injection and oviposition. On another date Hen 159, whose normal time between ovulation and oviposition was also 28 hours, was injected with labeled cells 9 hours prior to ovulation. This ovulation produced an egg containing a blood spot which did show detectable radioactivity, thus showing that the formation of the blood spot

occurred within that 9 hour period of time prior to ovulation. Further experiments using this method should show more accurately the time of blood spot formation. Blood spots formed within 48 hours after the labeled cells have been injected show detectable radioactivity if the blood spots contain more than 0.024 milliliters of blood.

Blood volume of the hen can be determined simultaneously by determining accurately the total radioactivity injected and then sampling the blood at 10 minute intervals after injection. The blood volume (V_t) can be calculated from the following formula:-

$$V_t = \frac{\text{(volume injected) (CPM/ml. injected)}}{\text{CPM / ml. sample}}$$

The volume of whole blood in a blood spot (V_{bs}) can be calculated by:-

$$V_{bs} = \frac{\text{CPM of blood spot}}{\text{CPM / ml. sample}}$$

CPM means counts per minute. An illustration using these formulas is as follows: Inject a hen with 2 milliliters of radioactive blood which showed 168,000 counts per minute per milliliter activity. If the withdrawn samples of blood after the injection had 2800 counts per minute per milliliter then the total blood volume (V_t) would be:-

$$V_t = \frac{(2 \times 168{,}000)}{2800} = 120 \text{ ml.}$$

If a blood spot had a total of 132 counts per minute, its volume

(V_{bs}) would be:-

$$V_{bs} = \frac{132}{2800} = .047 \text{ ml.}$$

All of the counts should be made at the same time to rule out the decay of the isotope.

The rate of disappearance of P^{32} from the blood appeared to follow a smooth hyperbolic curve as shown in Figure 1. The disappearance was most rapid during the first few hours of the first day after injection. This was probably due to the high excretion rate into the feces and eggs and the rapid removal of P^{32} by the reticuloendothelial system. The disappearance rate leveled off considerably by 5 days after administration and became ultimately tangent to the normal background level of blood radioactivity.

The disappearance of radioactivity in the feces following P^{32} injection is shown in Figure 2. Although some activity appeared in the feces within a few minutes after administration, the maximum radioactivity appeared in 15 to 24 hours. After this initial increase during the first day, a sharp irregular decline in activity followed for the next few days. Slight radioactivity continued for several weeks. This is in agreement with Shirley et al. (1951) who administered P^{32} orally.

Modifications of the initial technique were made during later trials. Since only a small percentage of the total P32

was absorbed by the red blood cells, much was left over in the supernatant mixture. A portion of this could be salvaged for similar experiments by adding it to other blood cells and repeating the incubation process. Eggs with blood spots were broken out into a petri dish and frozen at minus 40 degrees Fahrenheit. This aided in a clean removal of the blood spot from the yolk and prevented migration of the tracer.

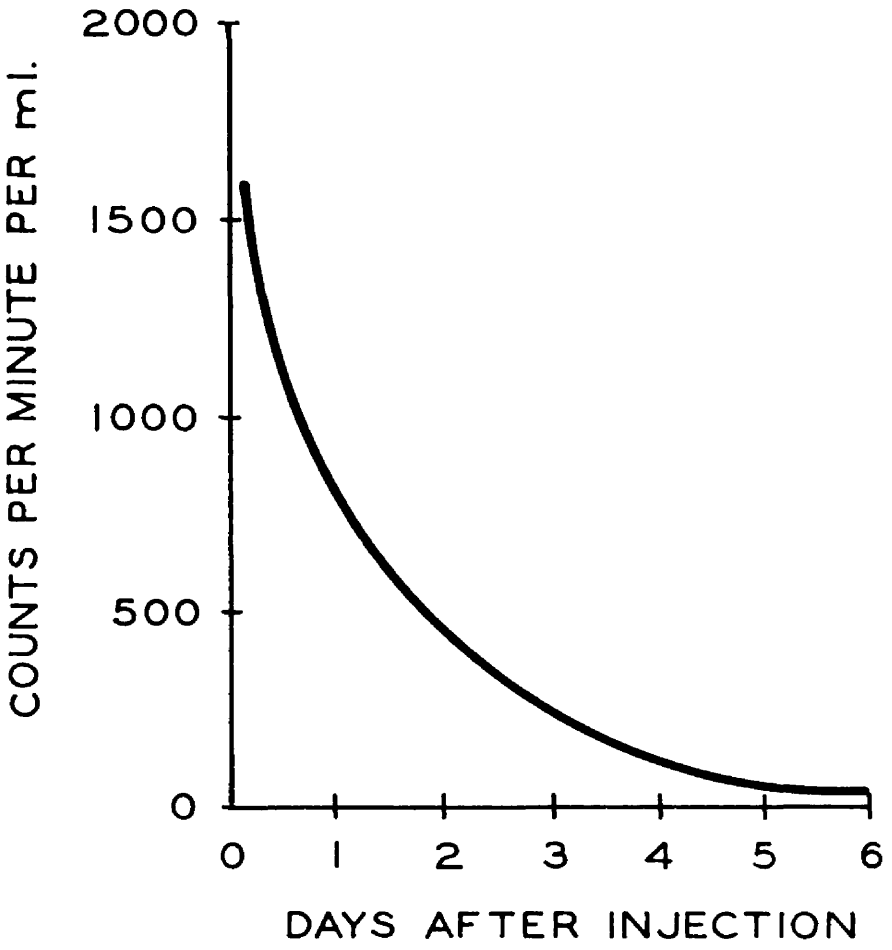

Figure 1. Loss of Radioactive Phosphorus from the Blood of a Hen.

TABLE 1

RADIOACTIVE PHOSPHORUS LOSS RATE IN THE BLOOD OF HEN 228

Sample	Amount ml	Total[1] count	Count per minute[1] per ml
Original p^{32}	0.001	8929	8,929,000
Original p^{32}	0.001	9441	9,441,000
		Ave.	9,185,000
Blood injected	0.1	19681	196,810
Blood injected	0.1	18657	186,570
Blood injected	0.1	19681	196,810
		Ave.	193,397
Blood 10 min. after inj.	0.4	769	1,922
Blood 10 min. after inj.	0.4	833	2,082
Blood 10 min. after inj.	0.4	817	2,042
		Ave.	2,015
Blood 20 min. after inj.	0.4	737	1,842
Blood 20 min. after inj.	0.4	849	2,122
Blood 20 min. after inj.	0.4	785	1,962
		Ave.	1,975
Blood 30 min. after inj.	0.4	753	1,882
Blood 30 min. after inj.	0.4	753	1,882
Blood 30 min. after inj.	0.4	785	1,962
		Ave.	1,909
Blood 24 hrs. after inj.	0.2	171	855
Blood 24 hrs. after inj.	0.2	192	960
Blood 24 hrs. after inj.	0.2	165	825
		Ave.	880
Blood 120 hrs. after inj.	0.5	16	32
Blood 120 hrs. after inj.	0.5	13	26
Blood 120 hrs. after inj.	0.5	8	16
		Ave.	25

1. Corrected for a background of 31 counts per minute.

TABLE 2

RADIOACTIVE PHOSPHORUS LOSS RATE IN HEN FECES;
FECAL COUNTS FROM HEN 228

Sample number	Sample weight grams	Hours after injection	Total counts per minute per gram[1]
1	0.5	2	337
2	1.0	15	808
3	1.0	39	475
4	1.0	63	501
5	1.0	87	65
6	1.0	111	71

1. Corrected for a background count of 37 counts per minute.

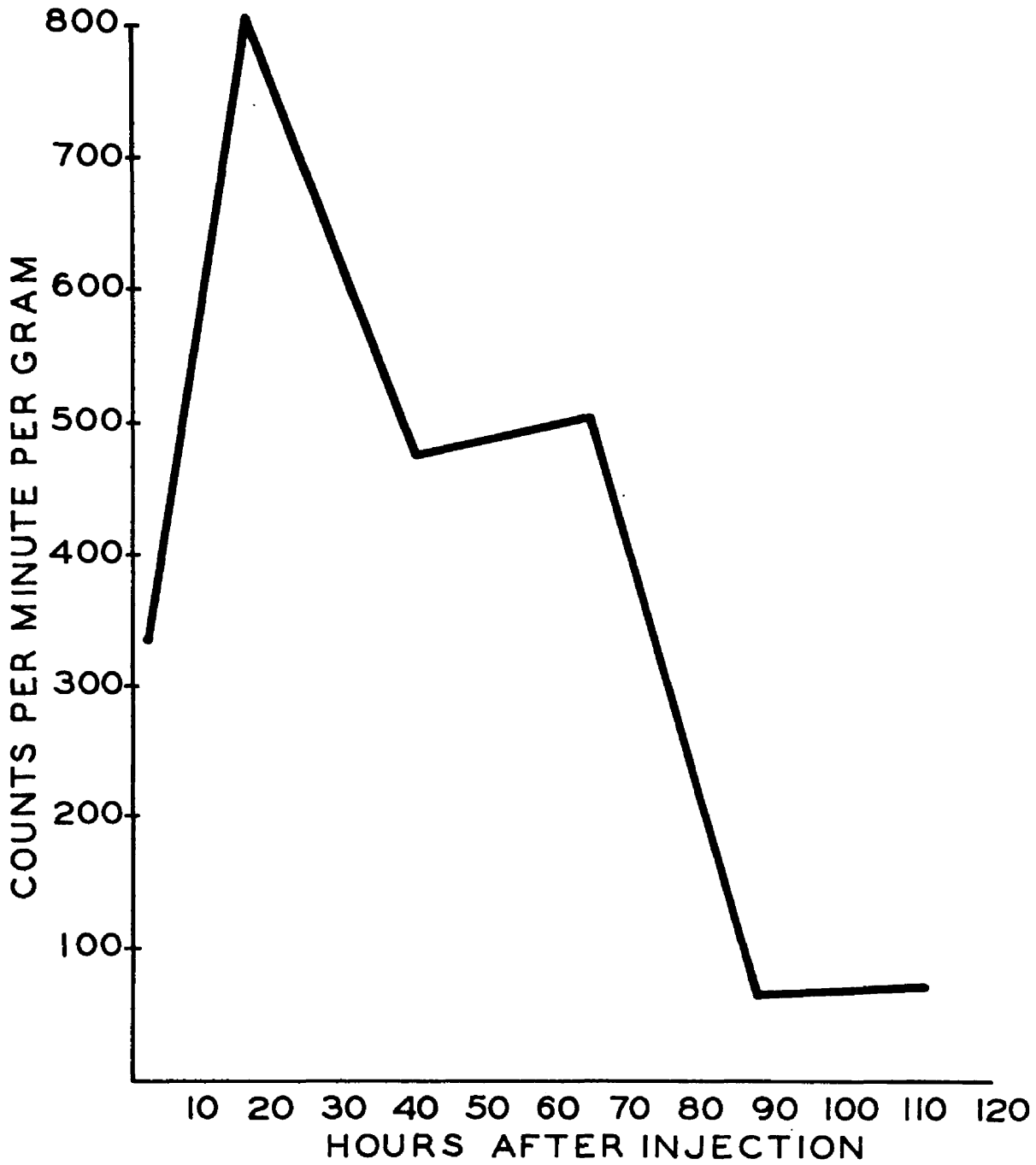

Figure 2. Radioactive Phosphorus Loss Rate in Hen Feces.

EXPERIMENT II. THE RELATIONSHIP OF OVIPOSITION TIME, CLUTCH POSITION, BAROMETRIC PRESSURE, AND EGG WEIGHT TO THE INCIDENCE OF BLOOD SPOTS IN EGGS

This experiment was designed to determine if the time of ovulation (based on oviposition time) was different for eggs containing blood spots than for normal eggs. In addition, the experiment was designed to discover if there was an influence on the incidence of blood spots due to egg clutch position, barometric pressure, egg weight and egg albumen height. These latter four conditions were studied because their effect (if any) could be investigated without altering the experiment's main objective, which dealt with the relationship of time of ovulation and oviposition to blood spot incidence.

Some studies of this nature have been reported in the literature, however, none were found to deal with the influence of ovulation time on blood spots. Quinn and Godfrey (1940) found no significant correlation between the percentage of blood spots and egg production, egg weight, or body weight. Jeffrey (1945) studied the relationship between the size and color of meat spots and the position of abnormal eggs in the clutch. He found that the percentage of large red meat spots was highest in the eggs in clutch position 1 followed by a sharp decline until a second peak was reached at positions 6, 7 and 8. Small meat spots were

most prevalent at the fifth clutch position. Strain and Johnson (1957) reported that eggs in the first clutch position had the highest incidence of blood spots and the second clutch position had the second highest incidence of blood spots. They found no significant influence of clutch position on meat spots.

Experimental Procedure

Eggs were collected hourly every day for 7 months from 61 yearling White Leghorn hens held in cages. These eggs were refrigerated (60 degrees Fahrenheit) until broken on a glass plate with mirrors both underneath and behind to allow full observation of the eggs for blood spots. All eggs were broken within 24 hours after they were laid, and oviposition time, weight, United States Department of Agriculture score[1], blood spots and other abnormalities were recorded daily. Albumen height, Haugh score and shell thickness were recorded 4 days a week. The accuracy of time the eggs were laid was within one hour, the egg weight was within one gram, the albumen height was within 0.1 milliliter, and the shell thickness was within 0.0001 inch. The size of each blood spot was differentiated as being large - over one-eighth of an inch in diameter, medium - between one-sixteenth and one-eighth of an inch in diameter, and small - less than one-sixteenth of an inch in diameter.

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1. An explanation of the United States Department of Agriculture score and Haugh score can be obtained from a publication prepared by Lester Kilpatrick, A. W. Brant and H. L. Shrader, 1958. Equipment and methods for measuring egg quality. AMS Number 246, U.S.D.A., Washington, D. C.

The barometric pressure data were obtained from the United States Weather Bureau in East Lansing. These data were acquired approximately one mile from where the chickens were housed. At this station the pressure was recorded at 7:30 a.m. and 1:30 p.m. Correlations were made between the pressure at these times with blood spots in eggs ovulated during the same time period and also in those ovulated at different time intervals until 2 days later. The data for this study included all of the pressure periods and all of the eggs from the hens on the experiment every day between December 12, 1956 through May 2, 1957.

Results and Discussion

Oviposition Time:-- The time between the laying of eggs within a clutch and between clutches was compared for normal eggs and eggs containing blood spots. Table 3 shows the results when all of the eggs were grouped in monthly units and these units analyzed statistically.[1]

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1. All the statistical procedures employed in this thesis were those of Walker and Lev (1953).

TABLE 3

THE AVERAGE TIME BETWEEN EGGS OF A CLUTCH AND BETWEEN CLUTCHES BASED ON MONTHLY AVERAGES

	Normal eggs		Blood spot eggs			
	Total eggs	Average time between eggs (hours)	Total eggs	Average time between eggs (hours)	Difference (min.)	Standard error (min.)
Between clutches						
1 day skip	1163	43.23	140	42.67	33.6*	18.0
2 day skip	88	69.45	9	68.67	49.3	34.8
Between eggs of a clutch						
2nd[1]	1109	26.57	91	26.02	33.0*	14.8
3rd[1]	713	26.72	42	26.08	38.4	25.9
4th[1]	375	26.08	7	25.93	9.0	43.4
All[2]	2635	26.34	172	25.79	33.0**	12.1

* Significant at the 5 percent level.

** Significant at the 1 percent level.

1. Refers to the time between this egg and the one immediately preceding it.

2. Refers to the average time between all of the eggs within the clutch regardless of clutch position.

The data in Table 3 reveal that the time interval between successive eggs in a clutch was less for eggs containing blood spots than for normal eggs. Where there were enough eggs for the data to be significant, the difference in the mean laying times averaged 33 minutes less to lay an egg containing a blood spot than to lay a normal egg. The standard error of the mean for the time in the one day skip between clutches, between the first and second egg in a clutch, and between all of the eggs within a clutch ranged from 12 to 18 minutes.

When the data were analyzed on an individual hen basis, the results were similar to those found when monthly averages were compared. These average individual hen comparisons are shown in Table 4.

Only the time comparisons for a one day skip between clutches, between the first and second egg of a clutch, and between all of the eggs within a clutch were analyzed on an individual hen basis. The average figure for each hen was treated as a unit and these units were analyzed statistically. A highly significant difference in oviposition time was found between blood spot eggs and normal eggs when the average times between eggs laid after a one day skip were compared and also when all eggs within a clutch disregarding clutch position were compared. These differences were 55.8 minutes for the one day skip and 48.0 minutes when all of the eggs were compared with

TABLE 4

THE AVERAGE TIME BETWEEN EGGS OF A CLUTCH AND BETWEEN CLUTCHES BASED ON INDIVIDUAL HEN AVERAGES

	Normal eggs		Blood spot eggs			
	Total eggs	Average time between eggs (hours)	Total eggs	Average time between eggs (hours)	Difference (min.)	Standard error (min.)
Between clutches						
1 day skip	937	43.01	109	42.08	55.8**	15.8
Between eggs of a clutch						
2nd[1]	931	26.31	74	25.57	44.4*	24.4
All	2317	26.48	147	25.68	48.0**	19.8

* Significant at the 5 percent level.

** Significant at the 1 percent level.

1. Time between this egg and the one preceding it.

the blood spot eggs requiring less time to be laid in both cases. When the oviposition times of eggs in the second clutch position were compared, the difference averaged 44.4 minutes less for blood spot eggs and was significant at the 5 percent level.

As reported in Table 4 the standard error was 15.8 minutes for the one day skip between clutches, 24.4 minutes for the time between the first and second egg of a clutch, and 19.8 minutes for the time between all of the eggs within a clutch.

Clutch Position:-- Since the birds were not trapped until 8:00 a.m. and lights were turned on at 4:30 p.m., it was suspected that the data might be slightly biased as the first egg of a clutch had a greater probability of being laid before 8:00 a.m. and had a greater probability of containing a blood spot than did the eggs of succeeding clutch positions. This was true in these data and agrees with the findings of Jeffrey (1945) and Strain and Johnson (1957). The percentages of blood spots found in eggs of the various clutch positions are reported in Table 5.

The Chi Square value of 25.64 reported in Table 5 was significant at the 1 percent level showing that the variation between the observed and the expected values in Table 5 were significantly different, that is, more blood spot eggs were found in the first clutch position and fewer in the third and fourth positions than could be accounted for on the basis of chance alone.

TABLE 5

THE PERCENTAGE OF BLOOD SPOT EGGS FOUND IN THE DIFFERENT CLUTCH POSITIONS

Clutch position	Normal eggs		Blood spot eggs		Percentage blood spot eggs
	Observed	Expected	Observed	Expected	
1	1118	1147.5	120	90.5	9.69
2	966	965.8	76	76.2	7.28
3	651	639.6	39	50.4	5.65
4	353	335.5	9	26.5	2.50
5	158	157.6	12	12.4	7.06

$X^2 = 25.64^{**}$

** Significant at the 1 percent level.

A Chi Square frequency test was performed on the data to determine if there was a significant difference between normal eggs and blood spot eggs laid at or before 8:00 a.m. These data are shown in Table 6.

TABLE 6

NORMAL EGGS AND BLOOD SPOT EGGS LAID BEFORE AND AFTER 8:00 am.

	Before 8:00 a.m.	After 8:00 a.m.
Normal eggs		
Observed	730	2774
Expected	747.01	2756.99
Blood spot eggs		$X^2 = 6.83$*
Observed	75	197
Expected	57.99	214.01

* Significant at the 5 percent level.

The Chi Square value showed that some bias existed as significantly more eggs containing blood spots were laid before 8:00 a.m. than one would normally expect. It is concluded from this, that the difference in the average time that blood spot and normal eggs are laid is a slight underestimate, in other words, the difference of 33 minutes shown in Table 3 is slightly

under the probable true difference. The differences ranging from 44.4 to 55.8 minutes in Table 4 are probably more accurate in accordance to the true difference because the system of analyzing these data put less emphasis on eggs laid prior to 8:00 a.m.

Barometric Pressure:-- Since changes in barometric pressure can have certain physiological effects on animals, this analysis was conducted to determine if there was a correlation between barometric pressure and the incidence of blood spots in eggs laid at various time intervals after the pressure was recorded. The time intervals used in the comparisons were for those eggs laid 24 to 28.5, 29 to 33, and 48 to 52 hours later.

The results, shown in Table 7, indicated that there was no significant correlation between high or low barometric pressures and the incidence of blood spots.

To determine if a difference occurred in the frequency of blood spots when the barometric pressure was increasing as compared to when it was decreasing, a Chi Square analysis was applied. The barometric pressure change was determined by the difference in pressure from 7:30 a.m. to 1:30 p.m. with this time range being the only one considered.

TABLE 7

CORRELATION BETWEEN BLOOD SPOTS AND BAROMETRIC PRESSURE

Time between the pressure period and when the eggs were laid (hours)	Correlation coefficient	t value[1]
24 - 28.5	.0156	.261
29 - 33	- .0183	.307
48 - 52	.013	.218

1. A t value of greater than 1.96 is required to be significant at the 5 percent level.

The results of the data shown in Table 8 indicated that increases or decreases in barometric pressure had no effect on the frequency of blood spots as none of the Chi Square values reached the significant level.

Egg Weight Variations:-- A comparison was made between the average weight of normal eggs and eggs containing blood spots during the month of May, 1957. Only the eggs laid in similar clutch positions were compared. Table 9 shows the results of this comparison.

The results as shown in Table 9 indicated that normal eggs weighed significantly less than did blood spot eggs when only eggs in the first clutch position were compared, and when all of the eggs disregarding clutch position were compared. There was no significant difference found in the egg weights

TABLE 8

THE EFFECT OF INCREASING AND DECREASING BAROMETRIC PRESSURE ON THE INCIDENCE OF BLOOD SPOTS

Barometric pressure change	Number of eggs with blood spots[2] 0	1	2	3	4	Chi Square value[1]
	24 to 28.5 hours after pressure change (days)					
Increasing	16	16	11	9	6	6.962
Decreasing	21	32	20	7	2	
	29 to 33 hours after pressure change (days)					
Increasing	35	15	6	2	0	1.044
Decreasing	45	24	9	3	1	
	48 to 52 hours after pressure change (days)					
Increasing	14	18	12	8	2	1.879
Decreasing	20	26	16	8	7	

1. A Chi Square value of 11.1 or greater is required to be significant at the 5 percent level.

2. The numbers in the body of the table are the frequency that the number of blood spot eggs were observed during each time period.

TABLE 9

A COMPARISON OF EGG WEIGHTS OF NORMAL EGGS AND BLOOD SPOT EGGS

Clutch position	Normal eggs Number	Ave. wt. (grams)	Blood spot eggs Number	Ave. wt. (grams)	Weight difference (grams)	Standard error (grams)
1	203	61.015	55	62.945	1.93**	0.316
2	169	61.059	22	60.273	.79	1.026
All	630	60.556	98	62.092	1.54**	0.345

** Significant at the 1 percent level.

when only the eggs of the second clutch position were compared. This analysis was done by using the modified t test comparing means. The standard errors were 0.316 grams for the first clutch position, 1.026 grams for the second clutch position, and 0.345 grams for all clutch positions.

Since eggs containing blood spots were ovulated sooner and weighed more than normal eggs, it was probable that the blood spot eggs were abnormal at or prior to ovulation or that a malfunction occurred in the reproductive organs which caused a hemorrhage of the capillaries and thus, blood spots were formed. There are probably other physical or biochemical abnormalities associated with this capillary fragility. As more of these factors and their associations become known, it will be easier to determine why intrafollicular bleeding forms blood spots.

The same eggs which were used for weight analysis were also measured for albumen height on a four day a week sample basis. No differences were observed in the albumen height of blood spot eggs and normal eggs.

EXPERIMENT III. THE EFFECT OF PHYSICAL DISTURBANCE, SOUND AND LIGHT ON THE INCIDENCE OF BLOOD AND MEAT SPOTS AND OTHER EGG QUALITY FACTORS

Research workers have been trying for many years to associate the incidence of blood and meat spots with various environmental factors that would influence a laying hen's emotions or in any other way result in the abnormal production of eggs. Jeffrey and Pino (1943) frightened birds with dogs and did not get an increase in the incidence of blood spots. Jeffrey (1945) found birds held in cages had a slightly higher blood spot incidence than did similar birds on the floor.

The environmental factors of physical disturbance, sound and light were studied in three separate phases in this experiment. It was thought that by keeping each of these phases separate and comparing different types of physical disturbance, sound and light, the exact nature of their influence, if any, could be studied. Although there are many other environmental factors such as temperature, humidity, oxygen and carbon dioxide levels that could be studied, it was felt that the three chosen were the most important. Physical disturbance causes both a muscular and emotional stress on the birds that might increase the blood pressure, heart rate, or certain hormone releases to the point that fragile capillaries in the ovaries are ruptured. Sound also causes an emotional stress greater than normal when

the sound is continuous or of an extreme pitch or intensity. Layers were subjected to intermittent high pitch sound waves, low pitch sound waves, and continuous sounds during the experiment's sound phase. Light in combination with activity affects the secretion of luteinizing hormone which in turn affects the ovulation cycle. Since the previous experiment indicated that both the time of lay and the position of the egg within a clutch influence blood spot incidence, it is conceivable that light, either intermittently or continuously, could directly affect intrafollicular bleeding.

Experimental Procedure

Five hundred yearling Dekalb hybrid hens were randomly divided into 4 pens of 125 birds. A schedule was set up to evaluate egg production and quality beginning with a no treatment control period, after which the various phases of the experiment were carried out in succession as follows: physical disturbance phase, no treatment control period, sound phase, no treatment control period, light phase, and a no treatment control period. Table 10 illustrates the overall plan of the experiment.

All of the birds were trapnested except in pen C of time period 2, which was the control pen for the physical disturbance phase. All of the eggs were broken and inspected for spots each

TABLE 10

THE DISTRIBUTION OF TREATMENTS BY TIME PERIODS

Time period	Date[1]	Pen A	Pen B	Pen C	Pen D
1	8/12 - 9/17	Control period			
2	9/18 - 9/28	Shake birds	Scare birds	Control	Normal trapping
3	9/30 - 10/14	Control period			
4	10/15 - 10/24	High whistle	Radio	Low whistle	Control
5	10/25 - 11/16	Control period			
6	11/19 - 12/20	15 min. light periods inter-mittent	24 hour light	Control	1.75 hours light 1.25 hours dark intermittent
7	12/22 - 1/8	Control period			

1. The period for the entire experiment was from August 12, 1957, to January 8, 1958.

day throughout the treatment period. During the periods between treatments eggs were broken and inspected only every second day. A random sample of 15 eggs per pen was measured on each day of breaking for albumen quality. All of the eggs were broken within 24 hours after laying and were examined for the presence of blood and meat spots. The breaking was done on a glass plate with mirrors both underneath and behind the glass plate to allow full vision of the broken out egg. The egg weight was measured within 1 gram, the United States Department of Agriculture score was based on the standard United States Department of Agriculture egg quality chart, the albumen height was measured with a micrometer within 0.1 millimeter and the shell thickness was measured with another micrometer within 0.0001 inches.

Time period 2 was the physical disturbance phase of the experiment. In pen A the hens were shaken mildly for about 20 to 30 seconds as they were removed from the trapnests. This caused them to ruffle their feathers with each shaking. The birds in pen B were chased around the pen three times a day by violently waving two large pieces of white cloth. No sounds were used and the birds were not handled other than during normal trapnesting. Each treatment period was from 3 to 5 minutes in duration. At the end of this period the hens appeared tired and accustomed to the chasing to the point that they would no longer fly up, but would still run from the chaser.

By the end of the 10th day of treatment the birds would not scare easily and would not fly up readily, but would still run when chased. Pen C was the non-trapnested control pen. These birds were not abnormally disturbed in any way. Pen D was the trapnested control pen. These birds were trapnested under normal conditions, and were not otherwise abnormally disturbed. During this period all of the eggs were saved and were broken for quality measurements and the presence of blood spots.

After a two week no treatment control period the sound portion of the experiment was initiated. In pen A, a common type silent dog whistle with a frequency above the human audible range was blown for a one minute duration once each hour from 7:30 a.m. until 4:30 p.m. each day. There was a slight sound leakage to pen B but not to the other pens. In pen B a radio was played medium loud (station WILS) for 24 hours per day. The treatment in pen C consisted of a low frequency whistle blown for a duration of one minute once an hour between 7:30 a.m. and 4:30 p.m. each day. Pen D was the control pen and had no abnormal sound administered. There was a large feed room between this pen and the other pens, thus preventing a sound leakage. A three week no treatment control period followed this phase of the experiment.

The light phase of the experiment followed the three week control period. Paper sacks were nailed over the windows and a small ventilation opening 6 inches wide was covered with burlap

to allow air circulation. The pens were all uniform in their window covering and ventilation. Two new 100 watt bulbs were placed in each of the four pens. The different light periods were switched on and off with electrical time switches. In pen A 56 light periods and 40 dark periods were administered. Each period was 15 minutes in duration for a total of 14 hours light and 10 hours of darkness. At certain intervals it was necessary to have 2 consecutive light periods due to a lack of flexibility in the time clock. No light period was longer than 30 minutes without an intervening dark period of 15 minutes. Lights were on 24 hours a day in pen B. Pen C was the control pen and had 14 continuous hours of light and 10 continuous hours of darkness during each 24 hour period. Pen D had alternating periods of 1.75 hours light with 1.25 hours of darkness to give a total of 14 hours of light and 10 hours of darkness during each 24 hour period. A two week non treatment control period followed the light phase of the experiment.

Results and Discussion

Egg Production:-- The variations in egg production during this experiment are shown in Tables 11, 12 and 13.

TABLE 11

EGG PRODUCTION PRIOR TO, DURING, AND FOLLOWING THE PHYSICAL DISTURBANCE PHASE

Time period	Days	Pen	Treatment	Eggs Produced		
				Total	Average no. per day	Percent
1	22	A	None	1427	64.86	55.68
1	22	B	None	1286	58.45	50.18
1	22	C	None	1489	67.68	57.85
1	22	D	None	1332	60.54	51.09
2	10	A	Shake birds	559	55.9	49.47
2	10	B	Scare birds	527	52.7	46.23
2	10	C	None	629	62.9	54.70
2	10	D	Normal trapping	555	55.5	47.03
3	8	A	None	434	54.25	48.01
3	8	B	None	422	52.75	46.27
3	8	C	None	467	58.38	50.77
3	8	D	None	401	50.12	42.48

TABLE 12

EGG PRODUCTION DURING AND FOLLOWING THE SOUND PHASE

Time period	Days	Pen	Treatment	Eggs Produced		
				Total	Average no. per day	Percent
4	10	A	High whistle	509	50.9	45.45
4	10	B	24 hour radio	462	46.2	37.02
4	8	C	Low whistle	417	52.12	46.13
4	10	D	None	468	46.8	40.34
5	11	A	None	479	43.54	38.88
5	11	B	None	476	43.27	37.96
5	11	C	None	465	42.27	37.74
5	11	D	None	439	39.91	34.40

TABLE 13

EGG PRODUCTION DURING AND FOLLOWING THE LIGHT PHASE

Time period	Days	Pen	Treatment	Eggs Produced		
				Total	Average no. per day	Percent
6	18	A	15 min. lt. per.	745	41.39	46.72
6	18	B	24 hours light	887	49.28	43.61
6	18	C	Control	888	49.33	44.94
6	18	D	1.75 hrs. lt. and 1.25 hrs. dark per 3 hours	762	42.33	37.46
7	8	A	None	330	41.25	36.83
7	8	B	None	402	50.25	46.10
7	8	C	None	423	52.88	47.21
7	8	D	None	348	43.50	38.84

At the beginning of the experiment the birds in all 4 pens produced eggs at a rate of over 50 percent with the birds in pen C having the highest production (57.85 percent) and those in pen B the lowest (50.18 percent). During the physical disturbance phase the birds in all the pens dropped from 3 to 6 percent in production with the treatment of shaking the birds as they were trapped showing the largest drop in production. The average percentage production from each pen during the different time periods is reported in Figure 3. This graph illustrates the general decline in egg production from the beginning of the experiment until time period 5; after which, there was a slight increase in egg production during time periods 6 and 7 except for the birds in pen A, which showed a decline during time period 7.

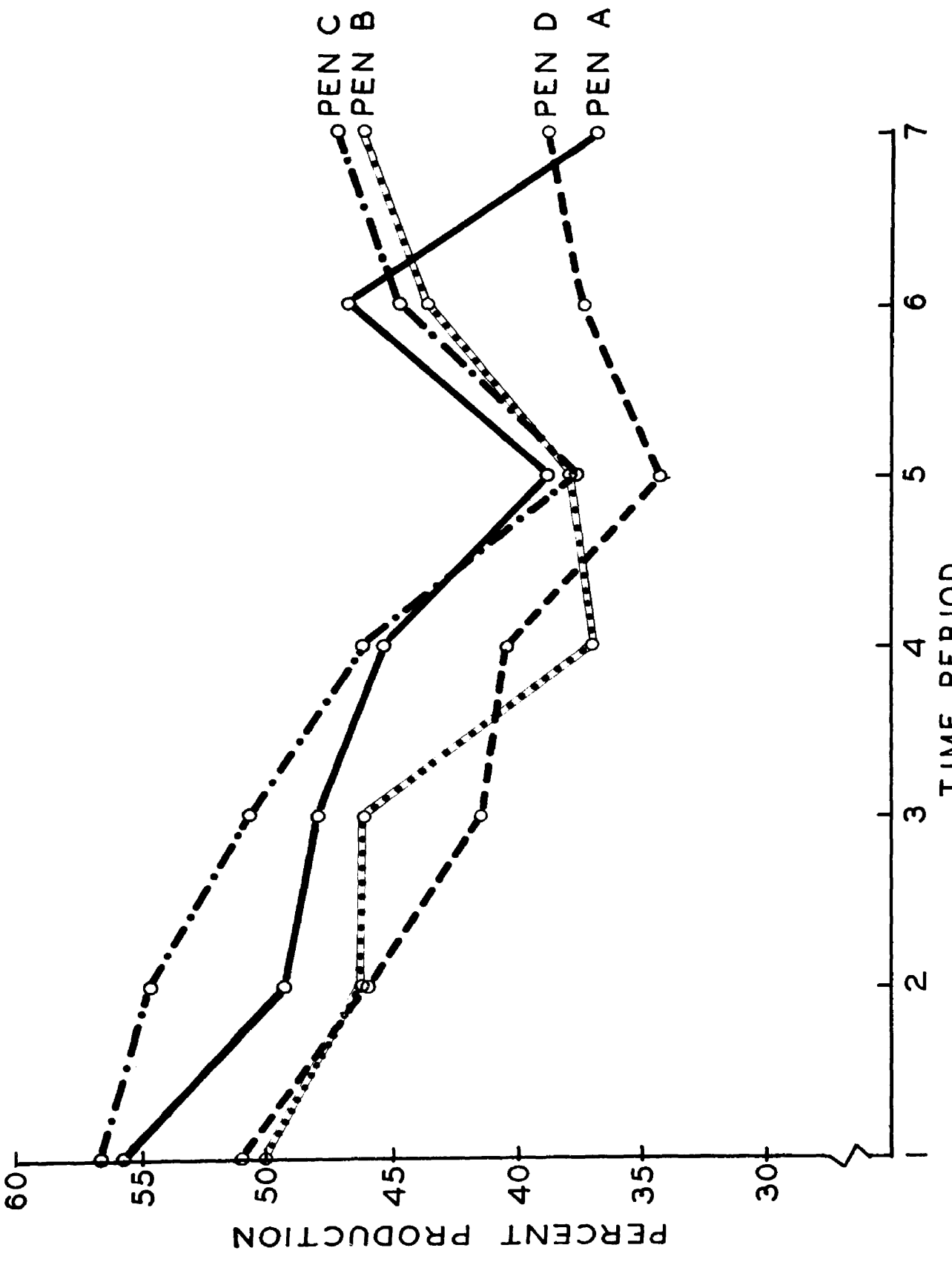

Figure 3. Egg Production During Experiment III.

Following the physical disturbance phase production continued to decline in all pens at approximately the same rate. The hens in pen B had a sharper decline in production in time period 4 than those in other pens and remained at a low level for the 3 week normal period following the 24 hour radio phase. It was doubtful that the radio had any major effect on this dip in production as birds in all of the other pens showed a similar trend. Several of the birds in these pens at this time had signs of respiratory infections, which was the probable cause for the decline in production during time periods 4 and 5. The rate of production increased in all pens during time period 6 and continued to increase slightly through time period 7 in all pens except for the birds in pen A. The evidence of respiratory infection was slightly more noticeable among the birds in pen A and probably accounted for the decline in production in the last period.

Blood and Meat Spots:-- The incidence of blood and meat spots in eggs obtained during time periods 1, 2 and 3 are shown in Table 14. These data were analyzed by analysis of variance and the means were compared for significant differences by the use of Duncan's multiple range test.[1] With this test, means on the same horizontal line in the tables are not significantly different while those on different horizontal lines are significantly different at the 5 percent level. The results of these

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1. Duncan, David B., 1955. Multiple range and multiple F tests. Biometrica 11:1-42.

analyses, where significant differences were found, are shown in Tables 15 and 16. The analyses where the F test was not significant are included in the appendix.

The percentage of blood spots among pens differed significantly during the control period (time period 1) prior to any treatments. This indicated that even though the hens were randomly distributed among the pens, variations greater than normal existed. The presence of a few hens laying a large number of blood spot eggs in one pen could easily cause this. The birds in pen A and D differed significantly from each other and also from pens B and C in the percentage of blood spot eggs.

The size of the blood spots were divided into large for those over one-eighth of an inch in diameter, medium for those between one-eighth and one-sixteenth of an inch in diameter, and small for those less than one-sixteenth of an inch in diameter. Statistical analyses were not applied to determine the differences between the various sizes among the pens as the variation was too great.

The brown meat spots were recorded, but were too few in number to be analyzed statistically. None of the treatments had any apparent effect on the incidence of brown meat spots. White meat spots were not recorded and none of the treatments had any noticeable effect on their incidence.

No significant differences were found among pens in the percentage of blood spots during the time period 2 (physical

TABLE 14

THE INCIDENCE OF BLOOD AND MEAT SPOTS PRIOR TO, DURING AND FOLLOWING THE PHYSICAL DISTURBANCE PHASE

	Pen			
	A	B	C	D
Time period 1				
Treatment	None	None	None	None
Total eggs	1427	1286	1489	1332
Blood spots				
Large[1]	38	24	24	20
Medium[1]	29	16	18	8
Small[1]	34	36	40	24
Total	101	76	82	52
Ave./day	4.59	3.46	3.76	2.36*
Percent	7.08*	5.91	5.51	3.90
Brown meat spots	8	2	3	5
Time period 2				
Treatment	Shake birds	Scare birds	None	Normal trapping
Total eggs	559	527	629	555
Blood spots				
Large[1]	17	16	15	14
Medium[1]	10	8	7	4
Small[1]	16	13	15	11
Total	43	37	37	29
Ave./day	4.3	3.7	3.7	2.9
Percent	7.69	7.02	5.88	5.22
Brown meat spots	4	2	0	0
Time period 3				
Treatment	None	None	None	None
Total eggs	434	422	467	401
Blood spots				
Large[1]	13	17	7	7
Medium[1]	9	5	3	1
Small[1]	8	11	11	4
Total	30	33	21	12
Ave./day	3.75	4.12*	2.62	1.5
Percent	6.91*	7.82**	4.50	2.99
Brown meat spots	3	3	2	2

* Significant at the 5 percent level.
** Significant at the 1 percent level.
1. Refers to the number of eggs having this size blood spot. The sizes are given in the discussion.

TABLE 15

ANALYSIS OF VARIANCE FOR PERCENTAGE BLOOD SPOTS DURING TIME PERIOD 1

Source	Degrees freedom	Sum of squares	Mean squares	F
Treatment	3	109.821	36.607	4.645**
Error	84	662.021	7.881	
Total	87	771.842		

Multiple Range Test

		(2)	(3)	(4)
Least significant range		1.686	1.776	1.836
Pen	D	C	B	A
Means (ranked)				7.077
		5.507	5.909	
	3.900			

** Significant at the 1 percent level.

TABLE 16

ANALYSIS OF VARIANCE FOR PERCENTAGE BLOOD SPOTS DURING TIME PERIOD 3

Source	Degrees freedom	Sum of squares	Mean square	F
Treatment	3	106.494	30.498	5.937**
Error	28	143.847	5.137	
Total	31	250.341		

Multiple Range Test

		(2)	(3)	(4)
Least significant range		2.323	2.435	2.507
Pen	D	C	A	B
Means (ranked)			6.912	7.820
	2.992	4.497		

** Significant at the 1 percent level.

disturbance phase); but the following time period, which was a no treatment control period, did show significant differences. The eggs from pens A and B had significantly higher blood spot incidence than those from pens C and D during time period 3, but these values were only slightly different from the values of eggs from the same pens during the previous period. It is the opinion of the author that even though the data show highly significant statistical differences, the differences are not necessarily due to the treatments, but are more likely due to the influence of a few hens laying a large number of blood spot eggs and that these few hens were not normally distributed. For any treatment to have a substantial effect, an increase of 10 percent or a decrease of 5 percent would appear to be a logical figure. For example, if the birds in a pen laid 10 percent blood spot eggs prior to any treatment and increased to 20 percent or decreased to 5 percent during a treatment period, then it could be assumed the treatment had a substantial effect.

The birds in all of the pens where sound was employed as a treatment were significantly higher in percentage of blood spots than those in the control pen during time period 4. These results are reported in Tables 17 and 18. Although the significant differences during time period 4 were partially due to a poor choice of a control pen, sound apparently did have a slight effect toward increasing the incidence of blood spots. The birds

TABLE 17

THE INCIDENCE OF BLOOD AND MEAT SPOTS DURING AND FOLLOWING THE SOUND PHASE

	Pen			
	A	B	C	D
Time period 4				
Treatment	High freq. whistle	24 hour radio	Low freq. whistle	None
Total eggs	509	462	417	468
Blood spots				
Large[1]	17	15	11	8
Medium[1]	11	9	13	5
Small[1]	15	21	14	9
Total	43	45	38	22
Ave./day	4.3*	4.5*	4.75*	2.2
Percent	8.45*	9.74**	9.11*	4.70
Brown meat spots	2	2	3	1
Time period 5				
Treatment	None	None	None	None
Total eggs	479	476	465	439
Blood spots				
Large[1]	7	13	12	4
Medium[1]	8	6	9	8
Small[1]	8	10	12	9
Total	23	29	33	21
Ave./day	2.09	2.64	3.00	1.91
Percent	4.08	6.09	7.10	4.78
Brown meat spots	4	3	2	9

* Significant at the 5 percent level.
** Significant at the 1 percent level.
1. Refers to the number of eggs having this size blood spot.

TABLE 18

ANALYSIS OF VARIANCE FOR PERCENTAGE BLOOD SPOTS DURING TIME PERIOD 4

Source	Degrees freedom	Sum of squares	Mean square	F
Treatments	3	136.53	45.51	3.881*
Error	34	398.655	11.725	
Total	37	535.185		

Multiple Range Test

		(2)	(3)	(4)
Least significant ranges		3.300	3.472	3.563
Pen	D	A	C	B
Means (ranked)		8.448	9.113	9.74
	4.701			

* Significant at the 5 percent level.

in all of the pens averaged an increased incidence during this time period, but all except those in the untreated control pen declined during the following no treatment control period. No particular type of sound appeared to have a greater effect than other types. There were no significant differences in blood spot incidence found during the following control period, which was time period 5.

The hens in pen B, which had lights burning continuously, laid eggs with a significantly higher percentage of blood spots than did hens from the control pen during the light phase (time period 6). The eggs in pen B had an increase of 2.37 percent blood spots during this period over the previous period. This indicated that lights burning continuously increased slightly the incidence of blood spots. The lack of uniform rest periods and the continual stirring about of some hens caused constant activity and stress within the pen and could account for the slight increase in blood spot eggs. The birds in pen A, which had the 15 minute alternating light and dark periods, also had an increase in blood spot incidence over the previous time period (3.30 percent). This increase was not significantly higher than those in the control pen. The longer intermittent light-dark periods in pen D did not have any effect on the number of blood spots. There were no significant differences in the incidence of blood spots in time period 7, which was the no treatment control

period following the light phase. These results are shown in Tables 19 and 20. The incidence of blood spots in eggs from the various pens during the entire experiment is shown in Figure 4.

Egg Quality:-- The egg quality data were analyzed by analysis of variance for the following factors: shell thickness, albumen height, egg weight, and Haugh score. No significant differences occurred in time period 1 for any quality factors, which indicated that the birds were uniformly distributed regarding these quality indicators. In the physical disturbance phase the birds that were chased 3 times a day had a significantly thinner shell, which averaged 0.31 thousandths of an inch less than eggs from the control pen. When the treatment ended the shell thickness increased. It was concluded from these data that frequently scaring birds has the effect of reducing shell thickness. No other quality factors were significantly influenced by the physical disturbance treatments. These results and statistical analysis are reported in Tables 21 and 22. The birds in the non-trapnested control pen had an average shell thickness of 13.40 thousandths of an inch and the birds in the trapnested control pen had an average shell thickness of 13.54 thousandths of an inch. The eggs from hens in pen B averaged 13.47 thousandths of an inch in time period 1, but then dropped significantly to 13.09 when the birds were scared 3 times a day in time period 2. The eggs from this pen increased to 13.59 thousandths

TABLE 19

THE INCIDENCE OF BLOOD AND MEAT SPOTS DURING AND FOLLOWING THE LIGHT PHASE

	Pen A	Pen B	Pen C	Pen D
Time period 6				
Treatment	15 min. light periods	24 hours light	Control	1.75 hrs.light 1.25 hrs. dark per 3 hours
Total eggs	745	887	888	762
Blood spots				
Large[1]	23	35	26	8
Medium[1]	22	14	7	13
Small[1]	10	26	15	18
Total	55	75	48	39
Ave/day	3.06	4.17**	2.67	2.17
Percent	7.38	8.46**	5.40	5.12
Brown meat spots	11	8	9	8
Time period 7				
Treatment	None	None	None	None
Total eggs	330	402	423	348
Blood spots				
Large[1]	8	15	6	8
Medium[1]	3	7	7	1
Small[1]	11	3	6	6
Total	22	25	19	15
Ave./day	2.75	3.12	2.38	1.88
Percent	6.67	6.22	4.49	4.31
Brown meat spots	1	6	3	4

** Significant at the 1 percent level.
1. Refers to the number of eggs having this size blood spot.

TABLE 20

ANALYSIS OF VARIANCE FOR PERCENTAGE BLOOD SPOTS DURING TIME PERIOD 6

Source	Degrees freedom	Sum of squares	Mean square	F
Treatments	3	124.643	41.548	4.413**
Error	68	640.146	9.414	
Total	71	764.789		

Multiple Range Test

		(2)	(3)	(4)
Least significant range		2.052	2.160	2.233
Pen	D	C	A	B
Means (ranked)			7.383	8.455
		5.405	7.383	
	5.118	5.405		

** Significant at the 1 percent level.

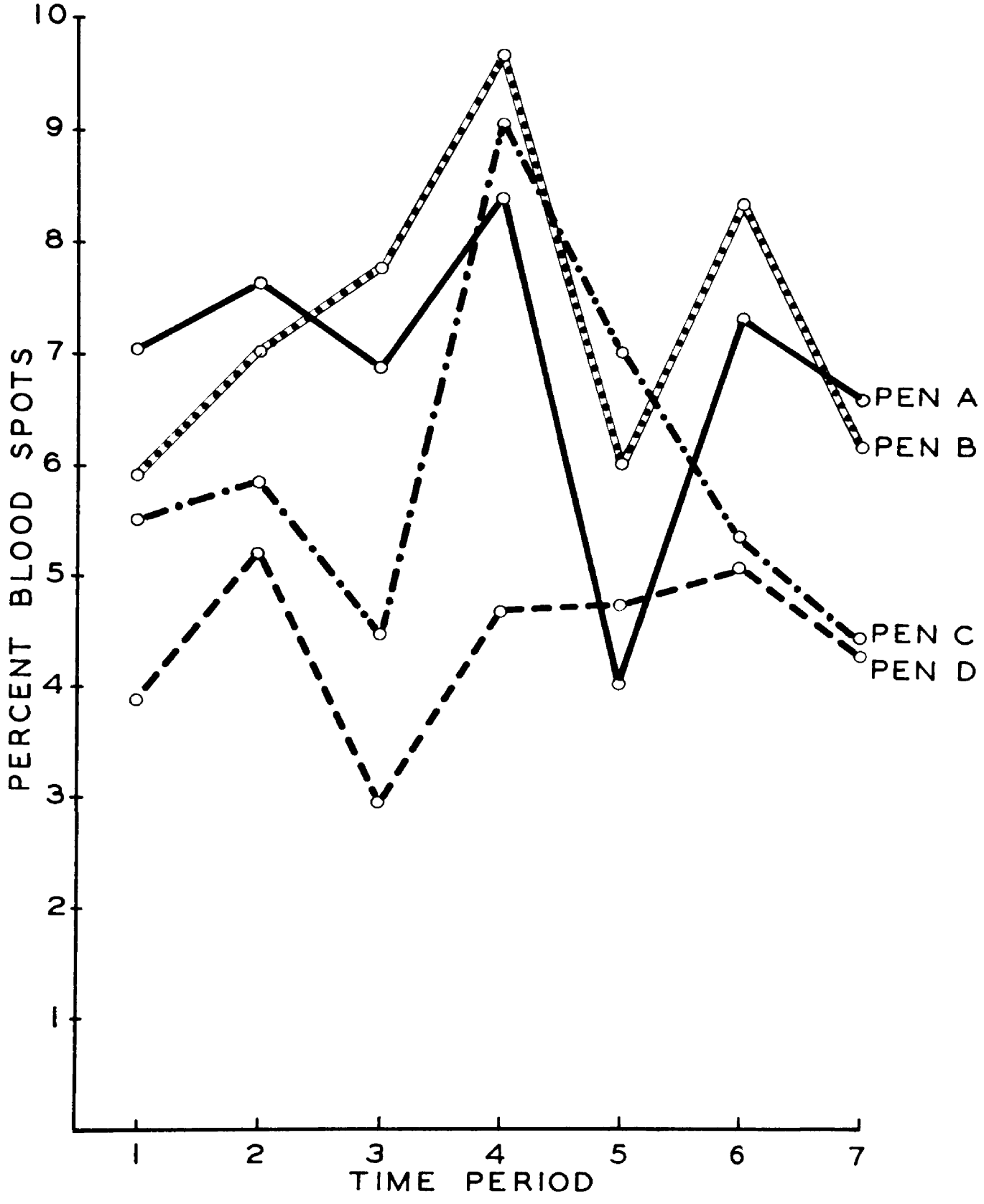

Figure 4. Percentage Blood Spots During Experiment III.

of an inch during the non treatment control period following time period 2. The eggs laid by birds that were shaken in pen A did not show any significant decline in shell thickness.

Only minor differences occurred among treatments in the average egg quality measurements during and following the sound phase, which was time period 4. Table 23 shows the mean values for each quality measurement during and following the sound phase. The statistical analyses are reported in Tables 24, 25 and 26. Where the data were not significant, the analyses are reported in the appendix.

The average albumen height of eggs from pen C (low frequency whistle) was significantly lower than the albumen height of eggs from pen A (high frequency whistle) and pen B (24 hour radio), but it was not significantly lower than the control pen in the same time period. The albumen height of eggs from pen C was significantly higher than the albumen height of eggs from pens A and B in the following no treatment control period (time period 5), but still did not vary significantly from eggs produced in the control pen. In this latter time period the mean Haugh score of eggs from pen C differed significantly from the mean Haugh score of eggs from pens A and B but not from those eggs observed in pen D, the control. These differences can be attributed to biological variation rather than to the treatment because the birds in pens with treatments did not differ significantly from the birds in the pen with no treatment.

TABLE 21

AVERAGE EGG QUALITY FACTORS PRIOR TO, DURING AND FOLLOWING THE PHYSICAL DISTURBANCE PHASE

	Pen			
	A	B	C	D
Time period 1				
Treatment	None	None	None	None
Shell thickness (.001 in.)	13.40	13.47	13.35	13.22
U.S.D.A. score	3.15	3.39	3.10	3.23
Albumen height (mm.)	7.24	6.96	7.29	7.11
Weight (grams)	62.09	61.79	61.98	61.80
Haugh score	83.95	81.96	84.26	82.94
Time period 2				
Treatment	Shake birds	Scare birds	None	Normal trapping
Shell thickness (.001 in.)	13.47	13.09**	13.40	13.54
U.S.D.A. score	3.40	3.44	3.33	3.25
Albumen height (mm.)	7.00	6.92	7.09	7.13
Weight (grams)	62.07	62.89	62.53	62.44
Haugh score	81.96	81.54	82.24	82.30
Time period 3				
Treatment	None	None	None	None
Shell thickness (.001 in.)	13.55	13.59	13.72	13.72
U.S.D.A. score	3.28	3.35	3.53	3.39
Albumen height (mm.)	7.12	6.99	6.82	6.91
Weight (grams)	63.40	64.48	63.41	63.14
Haugh score	83.30	81.21	80.36	81.12

** Significant at the 1 percent level.

TABLE 22

ANALYSIS OF VARIANCE FOR SHELL THICKNESS DURING TIME PERIOD 2

Source	Degrees freedom	Sum of squares	Mean square	F
Days	7	.305	.044	.475
Treatments	3	.946	.315	3.435*
Error	21	1.926	.092	
Total	31	3.177		

Multiple Range Test

		(2)	(3)	(4)
Least significant ranges		.317	.334	.340
Pen	B	C	A	D
Means (ranked)		13.389	13.471	13.537
	13.088	13.389		

* Significant at the 5 percent level.

TABLE 23

AVERAGE EGG QUALITY FACTORS DURING AND FOLLOWING THE SOUND PHASE

	Pen			
	A	B	C	D
Time period 4				
Treatment	High freq. whistle	24 hour radio	Low freq. whistle	None
Shell thickness[1]	13.71	13.86	13.64	13.63
U.S.D.A. score	3.47	3.47	3.83	3.63
Albumen height (mm.)	6.88	6.87	6.51	6.70
Weight (grams)	63.11	64.19	62.99	63.62
Haugh score	80.88	80.40	78.31	79.40
Time period 5				
Treatment	None	None	None	None
Shell thickness[1]	13.79	13.83	14.05	13.90
U.S.D.A. score	3.81	3.97	3.39	3.61
Albumen height (mm.)	6.48	6.34	7.00	6.72
Weight (grams)	63.48	63.04	62.58	62.96
Haugh score	77.87	76.52	81.50	79.51

1. Shell thickness was measured in 0.001 of an inch.

TABLE 24

ANALYSIS OF VARIANCE FOR ALBUMEN HEIGHT DURING TIME PERIOD 4

Source	Degrees freedom	Sum of squares	Mean square	F
Treatments	3	1.942	.647	8.087**
Error	30	2.398	.080	
Total	33	4.340		
	Multiple Range Test			
		(2)	(3)	(4)
Least significant range		.289	.304	.312
Pen	C	D	B	A
Means (ranked)		6.702	6.871	6.882
	6.509	6.702		

** Significant at the 1 percent level.

TABLE 25

ANALYSIS OF VARIANCE FOR ALBUMEN HEIGHT DURING TIME PERIOD 5 FOLLOWING SOUND PHASE

Source	Degrees freedom	Sum of squares	Mean square	F
Treatments	3	1.250	.417	5.024*
Error	16	1.332	.083	
Total	19	2.582		

Multiple Range Test

		(2)	(3)	(4)
Least significant ranges		.387	.406	.417
Pen	B	A	D	C
Means (ranked)			6.717	6.998
	6.340	6.478	6.717	

TABLE 26

ANALYSIS OF VARIANCE FOR HAUGH SCORE DURING TIME PERIOD 5 FOLLOWING SOUND PHASE

Source	Degrees freedom	Sum of squares	Mean square	F
Treatments	3	69.079	23.026	4.077*
Error	16	90.366	5.648	
Total	19	159.445		

Multiple Range Test

		(2)	(3)	(4)
Least significant ranges		3.42	3.59	3.68
Pens	B	A	D	C
Means (ranked)			79.511	81.497
	76.524	77.870	79.511	

* Significant at the 5 percent level.

During the light treatments, eggs from pens A, B and D differed significantly from eggs from the control pen (C) in albumen height and egg weight. Eggs from pens A and D varied significantly from eggs from the control pen in mean Haugh score. This definitely indicated that light had an effect on the albumen height and egg weight since eggs from the pens with the intermittent light and 24 hour light yielded significantly higher egg quality as determined by these measurements, than eggs from the control pen. It was not clear exactly how the different light exposure systems affected the albumen height or egg weight, but apparently the activity associated with the light periods influenced the secretion of thick albumen in the oviduct. Further research is needed to determine the exact influence light and activity have on egg quality characteristics. The results of the egg quality determinations during time periods 6 and 7 are given in Table 27. Tables 28, 29 and 30 show the statistical analyses for the significant portions of time period 6. The non significant analyses are given in the appendix.

No significant differences in egg quality were found among pens during the control period following the light phase.

TABLE 27

AVERAGE EGG QUALITY FACTORS DURING AND FOLLOWING THE LIGHT PHASE

	Pen			
	A	B	C	D
Time period 6				
Treatment	15 min. light periods	24 hours light	Control	1.75 hrs.lt. 1.25 hrs.dk.
Shell thickness[1]	13.86	13.91	13.89	13.60
U.S.D.A. score	3.64	3.70	3.97	3.54
Albumen height (mm.)	6.73**	6.69*	6.37	6.87**
Weight (grams)	64.58**	65.50**	62.60	63.84*
Haugh score	79.41*	78.59	77.03	80.18**
Time period 7				
Treatment	None	None	None	None
Shell thickness[1]	14.04	14.31	14.12	14.08
U.S.D.A. score	3.67	3.60	4.09	3.49
Albumen height (mm.)	6.73	6.82	6.25	6.78
Weight (grams)	65.20	64.09	64.25	62.29
Haugh score	79.12	79.98	75.32	80.29

1. Shell thickness measured in 0.001 of an inch.
* Significant at the 5 percent level.
** Significant at the 1 percent level.

TABLE 28

ANALYSIS OF VARIANCE FOR ALBUMEN HEIGHT DURING TIME PERIOD 6

Source	Degrees freedom	Sum of squares	Mean square	F
Treatments	3	1.72	.573	5.907**
Error	48	4.671	.097	
Total	51	6.391		

Multiple Range Test

		(2)	(3)	(4)
Least significant ranges		.245	.259	.266
Pen	C	B	A	D
Means (ranked)		6.692	6.726	6.871
	6.373			

** Significant at the 1 percent level.

TABLE 29

ANALYSIS OF VARIANCE FOR EGG WEIGHT DURING TIME PERIOD 6

Source	Degrees freedom	Sum of squares	Mean square	F
Treatments	3	58.653	19.551	9.970**
Error	48	94.140	1.961	
Total	51	152.793		

Multiple Range Test

		(2)	(3)	(4)
Least significant ranges		1.108	1.167	1.202
Pen	C	D	A	B
Means (ranked)			64.582	65.500
		63.84	64.582	
	62.598			

** Significant at the 1 percent level.

TABLE 30

ANALYSIS OF VARIANCE FOR HAUGH SCORE DURING TIME PERIOD 6

Source	Degrees freedom	Sum of squares	Mean square	F
Treatments	3	71.082	23.694	4.092*
Error	48	277.977	5.791	
Total	51	349.059		

Multiple Range Test

		(2)	(3)	(4)
Least significant ranges		1.90	2.01	2.07
Pen	C	B	A	D
Means (ranked)		78.593	79.414	80.183
	77.029	78.593		

* Significant at the 5 percent level.

DISCUSSION

Ovulation in the chicken occurs within 15 to 75 minutes after laying. It is apparently induced by the cyclic release of "luteinizing hormone" from the pituitary 6 to 8 hours earlier. The results of the first experiment in this study indicate that blood spots are formed approximately at the time of ovulation since the hen injected with P^{32}-labeled red blood cells prior to ovulation laid an egg containing a radioactive blood spot while a second hen, which was injected with P^{32}-labeled red blood cells after ovulation, laid an egg containing a non-radioactive blood spot.

The second experiment demonstrated that the average oviposition time for eggs containing blood spots is 33 to 50 minutes earlier than for normal eggs. It is reasonable to assume that the blood spotted ova might have been ovulated sooner than normal ova. Certainly there is no evidence that the incidence of blood spots bears any relationship to the time required for the ovum to pass through the oviduct. Since blood spots appear to be caused by unknown influences which culminate at approximately the time of ovulation, the statistical analyses of the second experiment and the experimental timing of the environmental factors studied in the third experiment were directed toward the time of ovulation, rather than toward the earlier pituitary activity. The barometric pressure data were correlated

with the incidence of blood spots at and prior to ovulation time. The results indicate that barometric pressure does not influence blood spot incidence.

A relationship between egg weight and blood spot incidence was shown to exist. Eggs containing blood spots are larger than normal eggs. Since egg weight is largely determined by yolk size, the implication is that a blood spot is more likely to occur when a larger yolk is ovulated earlier. This in itself could be the major mechanical cause of blood spots. Thus, the yolk in the first egg of a clutch is usually larger and has a greater probability of containing a blood spot than succeeding yolks. The data of the second experiment also show that the apparent ovulation time (based on the mean oviposition time) is earlier for the first yolk of a clutch if a blood spot is present.

Since "luteinizing hormone" (L.H.) influences ovulation time and follicle stimulating hormone (F.S.H.) influences ovum size, the time of their secretion by the pituitary might well be of major importance in blood spot production. Since light stimulates F.S.H. and general body activity inhibits L.H. secretion, other environmental stimuli might also influence the release of one or the other or both types of hormones. However, the ovulatory hormone (presumably L.H.) normally reaches its peak secretion at night, perhaps 6 to 8 hours before ovulation.

The third experiment was generally designed to determine

whether there is a direct effect of these environmental factors on blood spot incidence when the environmental stimulus is given during the day. In experiment III, day time physical disturbance had no effect on blood spot incidence. In order to influence the secretion of "luteinizing hormone", the disturbance would have had to take place at night. When sound was administered at night in pen B of time period 4, a highly significant increase in blood spot incidence over the controls occurred. A highly significant increase over the controls in blood spot incidence also occurred in the eggs from the pen which had lights burning continuously. In the light experiment, no significant differences in blood spot incidence occurred in eggs from pens having alternating periods of darkness. Further research is evidently needed to determine the influence of "luteinizing hormone" on blood spot incidence. In such experiments, external stimulation should coincide with the periods when the hens are in darkness and normally at rest.

In a few cases (scaring and intermittent light), significant effects of environmental stimuli could be observed in shell thickness and albumen characteristics but not in blood spot incidence. Apparently environmental factors can influence the secretion behavior of the various parts of the oviduct without influencing follicular functions of the ovary. Again, the endocrine basis for these observations requires further investigation.

The present incomplete data, however, all emphasize the significance of events happening in the ovary itself, rather than in other parts of the reproductive system, in relation to the problem of blood spot formation.

SUMMARY AND CONCLUSIONS

This study approached the blood spot incidence and egg quality problem from a physiological and environmental approach. Radioactive phosphorus was used as a tracer in the blood to follow the blood cells into the egg when a blood spot was formed. A technique whereby the red blood cells were labeled with radioactive phosphorus was adapted and made successful in labeling blood spots in chicken eggs. Although the transfer of radioactivity to the blood cells was relatively inefficient, substantial radioactivity was detected when the blood spots contained more than 0.024 milliliter of blood. Blood spots formed after the labeled cells were injected had radioactivity, while those formed prior to injection showed no activity. By knowing the time of injection and the time of ovulation, the period within which the blood spot was formed can be determined. By lengthening and shortening the time range in which blood spots are labeled, the time of blood spot formation can be more accurately predicted. By knowing what else took place within the hen's body at this time, the exact cause of this intrafollicular bleeding might become known.

A hen injected with P^{32}-labeled cells 3.5 hours after the predicted ovulation time laid an egg containing a blood spot that was not radioactive. This indicated the blood spot was formed

prior to the time of injection and before the ovum left the magnum. A second hen was injected with labeled cells 9 hours prior to the estimated time of ovulating an egg containing a labeled blood spot. It was concluded that this blood spot was formed within the 9 hour period between the time of injection and ovulation. This is in disagreement with the theory that blood spots are formed several days before ovulation. Further research is needed using this same technique to determine when meat spots are formed and to narrow the time range when blood spots are formed.

This blood labeling procedure may be used to determine the total blood volume in a hen and in a blood spot by comparing their counts per minute per unit of volume with the counts per minute of the volume injected. This has value in estimating the volume of small depositions of blood such as one finds in blood spots, but is not as economically feasible as other blood measuring methods when the total blood volume is desired. There is an inverse relationship between the quantity of radioactivity injected with the size of sample needed for study in that more radioactivity is required in the original injections when very minute spots or blood samples are observed than when larger ones are examined.

In Experiment II normal eggs were compared with eggs containing blood spots to determine if differences existed regarding

the time of oviposition, position in the clutch, and egg weight. In comparing normal and blood spot eggs of a similar clutch position, it was found that the mean average oviposition time of eggs containing blood spots occurred approximately 30 to 50 minutes sooner than normal eggs. The average time between the eggs of a clutch based on monthly averages was 26.34 hours for normal eggs and 25.79 hours for blood spot eggs. This difference of 0.55 of an hour or 33 minutes was highly significant indicating that whatever caused the blood spots to be formed might also cause ovulation to take place sooner than normal or that premature ovulation may be, in many cases, the cause of blood spot formation. However, this could not be true in all cases as some blood spot eggs are ovulated later than normal. The average time between the eggs of a clutch based on individual hen averages was 26.48 hours for normal eggs and 25.68 hours for blood spot eggs, which was a highly significant difference of 48 minutes. The difference of 48 minutes is probably nearer the true average difference than the 33 minutes because when the individual hen averages were compared, less bias would result as less emphasis was placed on eggs laid before 8:00 a.m. The first egg of a clutch has a greater chance of containing a blood spot than do succeeding eggs and also has a greater chance of being laid before 8:00 a.m.

No correlation was found between the barometric pressure

or net changes in pressure for the area and the incidence of blood spots in eggs laid 24 or 48 hours later. The hypothesis that changes in barometric pressure would influence the capillaries of the ovary in such a way that they might bleed proved to be false.

The mean weight of eggs containing blood spots was significantly greater than the mean weight of normal eggs when comparisons were made between eggs of the first clutch position and when all of the eggs were compared disregarding clutch position. This difference was 1.54 grams when all eggs were compared and 1.93 grams when only eggs of the first clutch position were compared with the blood spot eggs being heavier in both cases. Since the average blood spot egg is heavier but is ovulated sooner, one might hypothesize that a larger prematurely ovulated ovum is the result of some abnormal enzyme or hormone activity. A further study of the enzymes and hormones associated with ovulation is indicated to help solve the blood spot problem.

In Experiment III the effects of various types of physical disturbances, light, and sound on blood spot incidence and on other egg quality factors were studied. The physical disturbance consisted of shaking, scaring, and trapnesting birds. None of these treatments had a significant effect on egg production or blood and meat spot incidence during the disturbance phase.

However, significantly more blood spots were found in eggs from the scared and shaken birds during the following control period. This difference was attributed to a non-uniform distribution of high blood spot incidence hens rather than to the treatments because similar differences occurred prior to the treatments. Scaring the birds decreased the shell thickness significantly by 0.00031 of an inch, but did not seemto affect any other egg quality factors.

The average egg production from all pens, during and following the sound phase decreased, but this was not attributed to the treatments. In this phase, significant differences were found in the blood spot incidence among treatments with the eggs from the control pen having the lowest incidence. This was due, in part, to the choice of a control pen, which in this case was a pen of birds having one of the lowest blood spot incidences throughout the entire experiment. Eggs from all of the pens, including the control, had an increased blood spot incidence during the sound phase with eggs from the low frequency whistle pen showing the greatest increase. None of the sound treatments caused a substantial change in blood spot incidence or in other egg quality factors.

The light treatment caused little change in egg production with all pens showing a slight increase. The pen with continuous light had a significantly higher blood spot incidence

than the control pen. This difference appeared to be due to the treatment and not necessarily to non-uniformity among the pens. Eggs laid by birds given 15 minute intermittent light and darkness had a near significant increase in blood spot incidence. This, coupled with the results of continuous lighting suggests that failure to get a prolonged rest period might increase blood spot formation. Both continuous and intermittent light also increased albumen height, egg weight, and Haugh score. Apparently light plays an important role in producing quality eggs and better quality eggs can be obtained by using more controlled lighting periods than the normal 14 hours of light followed by 10 hours of darkness. More research is needed to determine the optimum number and length of light periods to get the highest quality and the most economical eggs.

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APPENDIX TABLE I

BLOOD SPOT - NORMAL EGG COMPARISONS FOR TIME OF LAY;
ONE DAY SKIP BETWEEN CLUTCHES

Month	Normal Eggs			Blood Spot Eggs		
	Total time (hours)	Total no. normal eggs	Ave. time/egg (hours)	Total time (hours)	Total no. blood spot eggs	Ave. time/egg (hours)
Dec.	5427.5	125	43.42	381	9	42.33
Jan.	8224.5	191	43.06	710.5	17	41.79
Feb.	9531	222	42.93	722.5	17	42.50
Mar.	9608.5	223	43.09	1222.5	29	42.16
Apr.	8222	189	43.50	953	22	43.32
May	6207.5	144	43.12	1586.5	37	42.88
June	3001	69	43.49	393.5	9	43.72
Total	50232	1163	302.61	5969.5	140	289.70
Average	7176	166	43.23	852.8	20	42.67

APPENDIX TABLE II

BLOOD SPOT - NORMAL EGG COMPARISONS FOR TIME OF LAY;
TWO DAY SKIP BETWEEN CLUTCHES

Month	Normal Eggs			Blood Spot Eggs		
	Total time (hours)	Total no. normal eggs	Ave. time/egg (hours)	Total time (hours)	Total no. blood spot eggs	Ave. time/egg (hours)
Dec.	1247.5	18	69.31	-	-	-
Jan.	684.5	10	68.45	136	2	68.00
Feb.	421.0	6	70.17	68	1	68.00
Mar.	1331.5	19	70.08	144	2	72.00
Apr.	1107.0	16	69.19	209.5	3	69.83
May	1100.5	16	68.78	65.5	1	65.5
June	210.5	3	70.17	-	-	-
Total	6102.5	88	4861.5	623.0	9	343.33
Average	871.8	12.6	69.45	124.6	1.8	68.67

APPENDIX TABLE III

BLOOD SPOT - NORMAL EGG COMPARISONS FOR TIME OF LAY; TIME BETWEEN THE FIRST AND SECOND EGG OF A CLUTCH

Month	Normal Eggs			Blood Spot Eggs		
	Total time (hours)	Total no. normal eggs	Ave. time/egg (hours)	Total time (hours)	Total no. blood spot eggs	Ave. time/egg (hours)
Dec.	2890	111	26.04	126.5	5	25.30
Jan.	5003	187	26.75	307.5	12	25.62
Feb.	5434.5	200	27.17	363.5	14	25.96
Mar.	5924	222	26.68	419.5	16	26.22
Apr.	4690	178	26.35	412.0	16	25.75
May	3963	150	26.42	500.0	19	26.32
June	1566	61	25.67	239.0	9	26.56
Total	29470.5	1109	185.08	2368.0	91	181.73
Average	4210.1	158.4	26.44	338.3	13	25.96

APPENDIX TABLE IV

BLOOD SPOT - NORMAL EGG COMPARISONS FOR TIME OF LAY; TIME BETWEEN THE SECOND AND THIRD EGG OF A CLUTCH

Month	Normal Eggs			Blood Spot Eggs		
	Total time (hours)	Total no. normal eggs	Ave. time/egg (hours)	Total time (hours)	Total no. blood spot eggs	Ave. time/egg (hours)
Dec.	2113	79	26.75	73.0	3	24.33
Jan.	3173.5	117	27.12	76.5	3	25.50
Feb.	2804	103	27.22	158.5	6	26.42
Mar.	3910	147	26.60	139.5	5	27.90
Apr.	3002	115	26.10	314.0	12	26.17
May	2819.5	106	26.60	231.5	9	25.72
June	1227.5	46	26.68	102.5	4	25.62
Total	19049.5	713	187.07	1095.5	42	181.66
Average	2721.4	101.9	26.7	156.5	6	25.96

APPENDIX TABLE V

BLOOD SPOT - NORMAL EGG COMPARISONS FOR TIME OF LAY; TIME BETWEEN THIRD AND FOURTH EGG OF A CLUTCH

Month	Normal Eggs			Blood Spot Eggs		
	Total time (hours)	Total no. normal eggs	Ave. time/egg (hours)	Total time (hours)	Total no. blood spot eggs	Ave. time/egg (hours)
Dec.	1009.5	39	25.88	-	-	-
Jan.	1266.0	47	26.94	24	1	24.00
Feb.	1223.0	47	26.02	28	1	28.00
Mar.	1883.0	71	26.52	52	2	26.00
Apr.	2043.5	79	25.87	28	1	28.00
May	1572.0	61	25.77	-	-	-
June	783.0	31	25.26	49.5	2	24.75
Total	97.80.5	375	182.26	181.5	7	130.75
Average	1397.2	53.6	26.04	36.3	1.4	26.15

APPENDIX TABLE VI

BLOOD SPOT - NORMAL EGG COMPARISONS FOR TIME OF LAY; TIME BETWEEN ALL OF THE EGGS WITHIN A CLUTCH

Month	Normal Eggs			Blood Spot Eggs		
	Total time (hours)	Total no. normal eggs	Ave. time/egg (hours)	Total time (hours)	Total no. blood spot eggs	Ave. time/egg (hours)
Dec.	8346	320	26.08	252.5	10	25.25
Jan.	10613.5	396	26.80	408	16	25.50
Feb.	10292.5	382	26.94	624.5	24	26.02
Mar.	13266	501	26.48	736	28	26.29
Apr.	11383.5	437	26.05	985	38	25.92
May	11125	425	26.18	1012.5	39	25.70
June	4495.5	174	25.84	439	17	25.82
Total	69522.5	2635	184.37	4457.5	172	180.50
Average	9931.8	376	26.34	636.8	24.6	25.79

APPENDIX TABLE VII

PRODUCTION OF TOTAL EGGS AND BLOOD SPOT EGGS DURING THE 7 TIME PERIODS OF EXPERIMENT III

Period	Pen	Treatment	Days checked	Production		Blood Spots		
				Total eggs	Ave.% prod.	Total no.	Ave. %	No. hens laying blood spots
1	A	None	22	1427	55.68	101	7.08	40
	B	None	22	1286	50.18	76	5.91	39
	C	None	22	1489	57.85	82	5.51	38
	D	None	22	1332	51.09	52	3.90	27
2	A	Shake birds	10	559	49.47	43	7.69	29
	B.	Scare birds	10	527	46.23	37	7.02	22
	C	No trapping	10	629	54.70	37	5.88	--
	D	Normal trap	10	555	47.03	29	5.22	23
3	A	None	8	434	48.01	30	6.91	19
	B	None	8	422	46.27	33	7.82	21
	C	None	8	467	50.77	21	4.50	16
	D	None	8	401	42.48	12	2.99	11
4	A	High freq. whistle	10	509	45.45	43	8.45	26
	B	24 hr. radio	10	462	37.02	45	9.74	23
	C	Low freq. whistle	8	417	46.13	38	9.11	30
	D	No sound	10	468	40.34	22	4.70	19
5	A	None	11	479	38.88	23	4.80	16
	B	None	11	476	37.96	29	6.09	16
	C	None	11	465	37.74	33	7.10	20
	D	None	11	439	34.40	21	4.78	16
6	A	15 min. light periods	18	745	46.72	55	7.38	31
	B	24 hrs. light	18	887	43.61	75	8.45	35
	C	14 hrs. light	18	888	44.94	48	5.40	31
	D	105 min. light	18	762	37.46	39	5.12	19
7	A	None	8	330	36.83	22	6.67	12
	B	None	8	402	46.10	25	6.22	18
	C	None	8	423	47.21	19	4.49	15
	D	None	8	348	38.84	15	4.31	12

APPENDIX TABLE VIII

ANALYSES OF VARIANCE FOR TOTAL BLOOD SPOTS

Source	Degrees freedom	Sum of squares	Mean square	F
Time period 1				
Treatments	3	55.671	18.557	5.726**
Error	84	272.227	3.241	
Total	87	327.898		
Time period 2				
Treatments	3	9.9	3.30	1.174
Error	36	101.2	2.81	
Total	39	111.1		
Time period 3				
Treatments	3	33.75	11.25	6.028**
Error	28	52.25	1.87	
Total	31	86.00		
Time period 4				
Treatments	3	39.879	13.293	3.906*
Error	34	115.700	3.403	
Total	37	155.579		
Time period 5				
Treatments	3	8.272	2.757	1.896
Error	40	58.364	1.459	
Total	43	66.636		
Time period 6				
Treatments	3	39.042	13.014	5.902**
Error	68	149.944	2.205	
Total	71	188.986		
Time period 7				
Treatments	3	6.844	2.281	.494
Error	28	129.125	4.612	
Total	31	135.969		

* Significant at the 5 percent level.
** Significant at the 1 percent level.

APPENDIX TABLE IX

ANALYSES OF VARIANCE FOR PERCENTAGE BLOOD SPOTS

Source	Degrees freedom	Sum of squares	Mean square	F
Time period 2				
Treatments	3	27.239	9.080	1.101
Error	36	296.793	8.244	
Total	39	324.032		
Time period 5				
Treatments	3	39.622	13.207	1.616
Error	40	328.011	8.200	
Total	43	367.633		

APPENDIX TABLE X

ANALYSES OF VARIANCE FOR SHELL THICKNESS

Source	Degrees freedom	Sum of squares	Mean square	F
Time period 1				
Days	6	1.114	.186	3.000
Treatments	3	.224	.075	1.210
Error	18	1.115	.062	
Total	27	2.453		
Time period 3				
Days	6	1.753	.292	3.244
Treatments	3	.169	.0563	.622
Error	18	1.614	.0896	
Total	27	3.536		
Time period 4				
Treatments	3	.292	.0973	.840
Error	30	3.476	.1158	
Total	33	3.768		
Time period 5				
Days	4	.666	.1665	.644
Treatments	3	. 161	.0536	.207
Error	12	3.102	.2585	
Total	19	3.929		
Time period 6				
Days	12	1.270	.1058	.898
Treatments	3	.834	.278	2.356
Error	36	4.268	.118	
Total	51	6.372		

APPENDIX TABLE XI

ANALYSES OF VARIANCE FOR EGG WEIGHT

Source	Degrees freedom	Sum of squares	Mean square	F
Time period 1				
Treatments	3	.431	.144	.163
Error	24	21.152	.881	
Total	27	21.583		
Time period 2				
Treatments	3	2.694	.898	.389
Error	28	64.640	2.308	
Total	31	67.334		
Time period 3				
Treatments	3	7.430	2.477	1.260
Error	24	47.196	1.966	
Total	27	54.626		
Time period 4				
Treatments	3	7.591	2.530	1.417
Error	30	53.543	1.785	
Total	33	61.134		
Time period 5				
Treatments	3	2.075	.692	.638
Error	16	17.352	1.084	
Total	19	19.427		

APPENDIX TABLE XII

ANALYSES OF VARIANCE FOR HAUGH SCORE

Source	Degrees freedom	Sum of squares	Mean square	F
Time period 1				
Treatments	3	22.846	7.615	2.80
Error	24	65.293	2.720	
Total	27	88.139		
Time period 2				
Treatments	3	2.870	.957	.182
Error	28	146.943	5.248	
Total	31	149.813		
Time period 3				
Treatments	3	13.426	4.475	.403
Error	24	266.735	11.114	
Total	27	280.161		
Time period 4				
Treatments	3	30.539	10.180	2.767
Error	30	110.370	3.679	
Total	33	140.909		