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EVALUATION OF THE OVULATED FOLLICLE TECHNIQUE AS A MEANS OF DETERMINING PHEASANT PRODUCTION

BY

C. DENIS ALLEN

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Wildlife Biology, South Dakota
State University

1969

EVALUATION OF THE OVULATED FOLLICLE TECHNIQUE AS A MEANS OF DETERMINING PHEASANT PRODUCTION

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Advisor — Date

Head, Wildlife and Fisheries Sciences Department — Date

EVALUATION OF THE OVULATED FOLLICLE TECHNIQUE AS A MEANS OF DETERMINING PHEASANT PRODUCTION

Abstract

C. Denis Allen

A study of the ovulated follicle technique for determining egg production in pheasants was conducted during 1967 - 1969.

Evidence indicated collection of wild hens for follicle counts should be between 5 and 14 weeks after cessation of laying.

Questionable follicles encountered should be considered ovulated follicles and included in the counts. Most questionable follicles were believed to originate from ovulated atretic follicles that were harder to identify because of their particular stage of regression.

The influence of freezing on atretic follicle counts was studied, and no adverse effects were found.

A technique was developed for selectively staining atretic follicles. Faded or obscure atretic follicles were more easily identified when stained. Counts of ovulated atretic follicles from stained material were significantly more accurate than counts from unstained material.

The accuracy of the technique was sufficiently demonstrated to justify its use for the prediction of mean egg production. Variability between counts of different investigators was not significant.

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INTRODUCTION

The nature of wildlife management makes it essential that the biologist base his understanding and recommendations on knowledge gained through technology. Direct observation is often impossible and information must be gathered by using techniques designed to measure natural occurrences. If the biologist is to correctly understand and evaluate nature his techniques must be sound. In order for a technique to be sound it must demonstrate validity by measuring what it is intended to measure and it must be reliable.

A technique used to determine the number of eggs laid during the breeding season is to count the atretic follicles on the ovary. Meyer et al. (1947), Kabat et al. (1948), and Buss et al. (1951) introduced the technique of counting atretic follicles of pheasant ovaries to determine the number of eggs ovulated during the breeding season. After ovulation, the follicular sheath that formerly surrounded the yolk remains attached to the ovary (VanTienhoven 1959, Romanoff and Romanoff 1949). Since each atretic follicle represents an ovulated yolk, the number of eggs laid could in theory be determined by counting these follicles.

Atretic follicles are those in the process of atrophy or resorption (Rowan 1930) and may be either ovulated or unovulated (Romanoff and Romanoff 1949). Atresia of ovulated follicles is

characterized by decrease in size and an increase in the degree of coloration by red pigments (Meyer et al. 1947, Kabat et al. 1948, and Buss et al. 1951). After ovulation the follicle is resorbed at a rapid rate for several days until all that remains is a small pigmented remnant (Meyer et al. 1947). After 7 to 8 days regression becomes very slow and the small pigmented remnant may persist for several months. How long these remnants can still be recognized and correctly counted is unknown.

Different degrees of pigmentation of atretic follicles have been noted by various authors. Meyer et al. (1947) described them as brownish-orange while Greb (1962) observed reddish to orange-yellow remnants. These differences in pigmentation cause confusion as to what structures actually constitute an atretic follicle. It is not known whether the questionable follicles which result from this confusion should be included in counts.

It is also necessary to determine whether counts from individual hens correlate to their known laying records. In earlier studies by Meyer et al. (1947), Kabat et al. (1948), and Buss et al. (1951), a number of hens were kept together in large cages making individual comparisons impossible. Also, ground cover in the cages did not permit the finding of all eggs.

In order to clarify some of these ideas objectives of this study were designed to:

(1) Evaluate the ovulated follicle technique as a means of determining egg production by using hen pheasants with known

laying records..

(2) Determine the effects freezing of ovaries has on ovulated atretic follicle counts.

(3) Compare counts of questionable but probable atretic follicles as well as obvious atretic follicles with the known egg-laying record of hen pheasants.

(4) Employ various staining techniques to determine if ovulated atretic follicles can be more reliably recognized when stained.

(5) Study regression of ovulated follicles to determine how long after egg laying ceases the technique of counting ovulated atretic follicles is reliable.

METHODS AND MATERIALS

Collection and Dissection of Ovaries

Ovaries were obtained from adult hen ring-necked pheasants, (*Phasianus colchicus*), used in a previous study at South Dakota State University. The hens were kept in small individual cages and a daily laying record was kept for each one.

Hens were sacrificed at the end of the experiment and their ovaries removed immediately and stored in FAA (5 parts 95% alcohol, 1/2 part glacial acetic acid, 1 part commercial formalin, and 3 1/2 parts water). It was necessary to leave the ovaries in FAA for at least 3 days to fix the tissue and harden it sufficiently for dissection. In order to study effects of freezing on atretic follicle counts, hens that died during the experiment were frozen until needed. Frozen hens were allowed to thaw about 12 hours before ovaries were removed and placed in FAA.

Ovaries were removed from hens by making a transverse cut on the left ventral side extending from base of sternum to rib cage. By pushing abdominal viscera to one side the ovary was located in its dorsal midline position on the ventral surface of the kidney. The ovary was removed by carefully pulling it away from underlying kidney tissue with a forceps. To avoid tearing the ovary it was often necessary to remove it with part of the dorsal aorta, especially in cases where the ovary was small.

Figure 1 shows a representative ovary from a non-laying hen and Figure 2 an ovary from a hen that laid a number of eggs.

A low power (7x) dissecting scope was used for dissecting and examining ovaries. A more powerful scope was found to restrict the field of view, making dissection very difficult.

Before dissection ovaries were rinsed in a small bowl of water to remove the FAA. After removing the ovary from the rinse it was placed on the stage of the scope and flooded with water. The ovary was moistened several times during dissection to keep it from drying out.

All extraneous material was removed by carefully picking it away with a pair of forceps. If the aorta was tightly adhered, it was left attached. Any large unovulated follicles were then removed by plucking with a pair of forceps. Care was taken to examine the base of these follicles near their point of attachment for any small atretic follicles (Fig. 3). If no atretic follicles were present on large unovulated follicles, they were discarded.

After all large unovulated follicles were removed, the ovary was ready for dissection. Atretic follicles were usually obscured by many small non-ovulated follicles making it necessary to dissect the ovary into smaller pieces and examining these individually. This was accomplished by carefully working and pulling it apart with a pair of forceps. After counting each piece it was put aside in a water-filled dish to keep it separate from the rest

Figure 1. Ovary from hen that did not lay.
(Scale divisions = 1mm.)

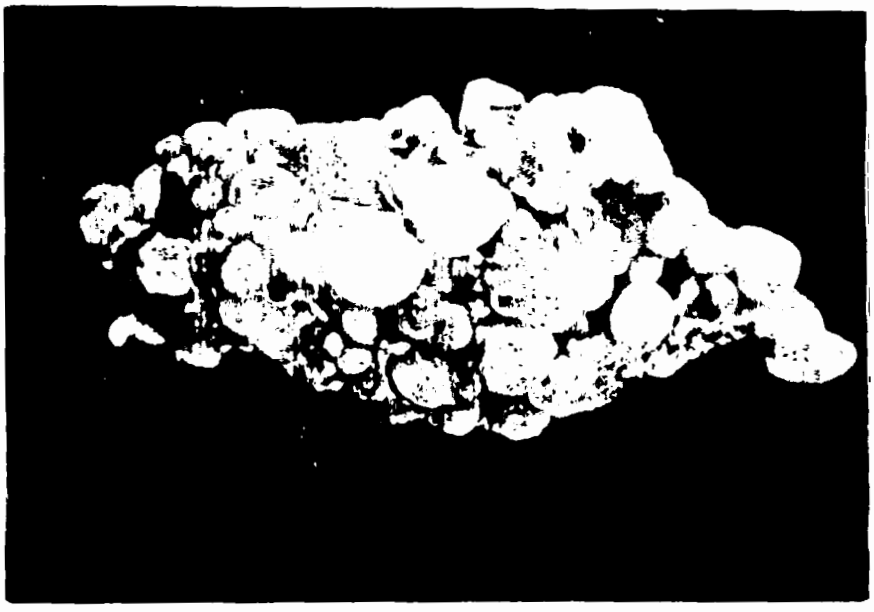

Figure 2. Ovary from hen that laid a number of eggs.

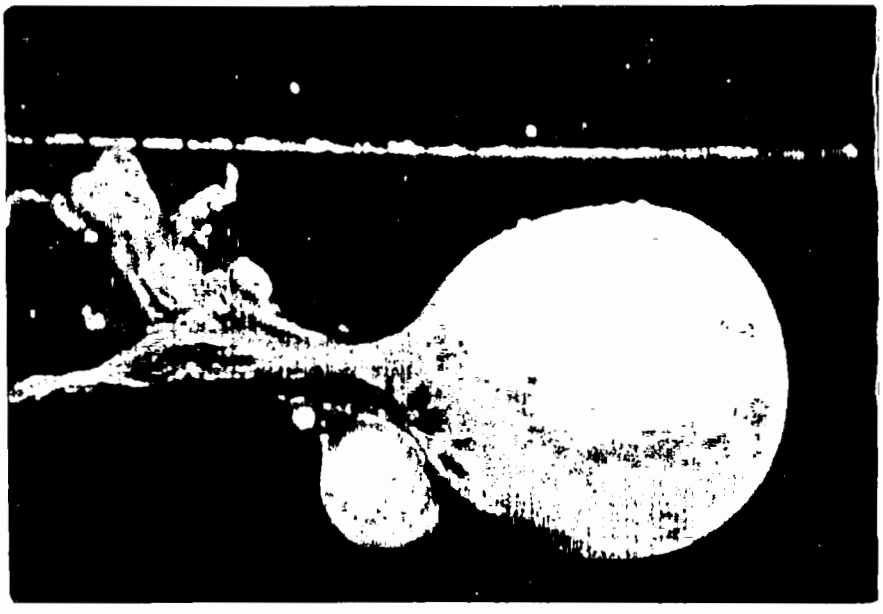

Figure 3. Large unovulated follicle with atretic follicle attached near its base.

of the material. After completing the count this material was stored in FAA and saved for future recount.

Analysis of Atretic Follicle Counts

Data obtained from follicle counts were compiled into two groups for analysis; one including and the other excluding questionable material (data obtained from individual hens are listed in the Appendix). Within these two groups comparisons were made between laying records and follicles counted using the paired "t" test (Steel and Torrie 1960).

Data including questionable follicles were further divided into frozen and unfrozen material. Comparison was then made between these two components and the known eggs laid for each group using the paired "t".

Different stages of follicular regression, and their effects on follicle counts were studied by dividing the total sample into two groups; one having a short, the other a long regression period. Regression time was considered to be the period between the last egg laid, and the death of the hen. Twenty-four hens, with a regression time 2 - 23 days, were selected as one regression group, and 32 hens having regression times 54 - 138 days comprised another group. Comparisons were made within each group using the paired "t".

Four groups of data obtained from ovaries having different regression periods of 1 - 10 days, 15 - 60 days, 65 - 100 days

and 100 - 125 days were tested with a completely randomized design. Each group consisted of counts from 12 ovaries. Orthogonal comparisons were made between these groups to find any significant differences existing between them.

In an attempt to determine variability existing between investigators, four people were recruited to count ten selected ovaries. These investigators were shown color photographs of atretic follicles in various stages of regression and methods used in counting were explained to them. Known eggs laid were subtracted from follicle counts obtained for each ovary, and differences were analyzed according to methods given by Steel and Torrie (1960) for analysis of variance for any number of groups with equal replication.

Color Reactions and Staining

Both color reactions and stains were tested in an attempt to develop a selective staining technique for ovulated atretic follicles.

The color reaction techniques used included the Schultz method for determining cholesterol (Thompson and Hunt 1966) and the color change caused by the reaction of carotenoid pigments and related compounds with strong acids (Rosenheim and Drummond 1925, Thompson and Hunt 1966). The latter procedure involved washing the ovarian tissue in distilled water to remove excess FAA and blotting it dry on a paper towel. Then,

while observing the material through a binocular dissecting scope for any color change, a drop of concentrated acid was added (either concentrated sulphuric, hydrocholoric, or perchloric acid).

Several fat stains that have an affinity for carotenoid pigments present in ovulated follicles were also tested. Sudan Black B, Sudan III, Sudan IV, and Oil Red O were mixed with propylene glycol as described by Thompson and Hunt (1966).

Unstained tissue was dissected into smaller pieces as described above, rinsed in water for 5 minutes, blotted dry on a paper towel, and placed in propylene glycol for 10 minutes. The excess propylene glycol was drained onto a paper towel, and the tissue put in stain for one hour after which it was placed in destaining solution.

Full strength solutions of ethyl alcohol, isopropyl alcohol, methyl alcohol, and acetone were tried in an attempt to find a solution that would properly destain the ovarian tissue. In addition, acid ethyl alcohol and 1 : 1 solutions of acetone, ethyl alcohol, and isopropyl alcohol with water were used.

To establish optimum staining time, tissues were stained for periods of 5 1/2, 2, 1, and 1/2 hours. These periods were then evaluated by the amount of time required to destain the tissue from each, and how well atretic follicles were stained. Enough time had to be allowed for atretic follicles to become

adequately stained, yet not so long that other ovarian tissue overstained.

After the technique for selectively staining ovulated atretic follicles was established, accuracy of this technique was tested by counting the atretic follicles of 23 unstained ovaries then staining the ovaries with Oil Red O. Material was destained in a 1 : 1 solution of isopropyl alcohol and water changed after 24 hours. A minimum of 48 hours was required to properly destain ovaries and frequently more time than this was needed. Material was considered properly destained when non-follicular ovarian tissue was left almost completely unstained. Data obtained from counts before and after staining were compared with known eggs laid using the paired "t".

RESULTS AND DISCUSSION

Questionable Follicles

This study indicated questionable follicles should be included in counts of atretic follicles. Comparison between follicle counts, including questionable follicles, and eggs laid indicated no significant differences between counts ($t = 1.890$, $P > 0.05$). When the number of eggs laid were compared to counts in which questionable follicles had been excluded, a highly significant difference was detected ($t = 3.750$, $P < 0.05$). The questionable follicles were needed in counts to fully account for eggs laid. Observations indicated questionable follicles were ovulated follicles in a particular stage of atresia.

The possible origins of questionable follicles could be caused by poor technique, unovulated atretic follicles, ovulated atretic follicles or a combination thereof.

A minimum number of questionable atretic follicles are present even in the most carefully dissected ovaries. Questionable follicles from this source may or may not be of ovulated follicle origin. Fat tissue can be mistaken for atretic follicles if mutilated in dissection because both are identically colored (Fig. 4). Also, unovulated follicles can be mistaken for recently ovulated follicles if torn. Careless techniques resulting in large numbers of questionable atretic follicles of non-follicular origin can lead to a significant amount of error in the counts.

Figure 4. Comparison showing the identical coloration of fat tissue (under pointer) and atretic follicle (center).

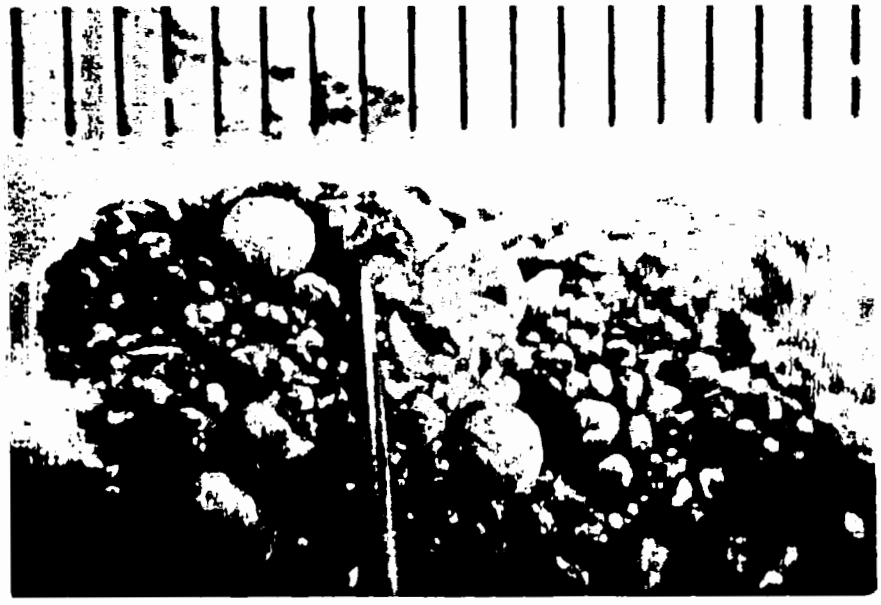

Figure 5. Pigmented unovulated atretic follicle on ovary.

It is not known to what degree atresia of unovulated follicles takes place in pheasant ovaries or if these occurrences contribute significantly to questionable follicles. Meyer et al. (1947) believed that only a small percentage of follicles counted could be attributed to this source. Rowan (1930) suggested, however, that atresia of unovulated follicles may be more common than suspected, but not readily observable. Pheasants are indeterminate layers and egg development is continuous until the clutch has been completed and laying stops. Developing follicles that have not been ovulated remain on the ovary at the cessation of laying. Large quantities of yolk must be resorbed from these ova indicating that some form of atresia must occur even though ovulation has not taken place. Atresia of these large unovulated follicles has been described in the South American cowbird by Davis (1942). Instances of this type of atresia were also observed in this study (Fig. 5).

Atresia of unovulated follicles exists but may not cause questionable follicles that could introduce error into counts. Davis (1942) noted unovulated atretic follicles of the South American cowbird bore close resemblance in later stages to ovulated ones; however, they could be separated for some time after ovulation. Kabat et al. (1948) stated that with few exceptions unovulated atretic follicles did not show pigmentation characteristic of post ovulatory follicles. In this

case the later stages of unovulated atretic follicles would also be expected to be unpigmented, and confusion with ovulated atretic follicles would be avoided. Unovulated follicles in the first stages of atresia, that were examined in this study, were well pigmented (Fig. 5). Later stages of these follicles will also probably be pigmented, in which case they are likely to be confused with post-ovulatory follicles. When wild birds have renested and laid several clutches, the number of follicles that may have gone through atresia at the cessation of each laying period could be large enough to inject serious error into counts. Further work is needed to determine the extent of error follicles of this type produce.

Ovulated atretic follicles pass through three distinct stages of atresia as described below. During one of these stages atretic follicles are particularly hard to identify. As a result of this, they become the largest known source of questionable follicles.

For approximately one week after ovulation, follicles are large (up to 30 mm. in length), have distinguishing features and are easily counted. They resemble the hull of a grape from which the pulp has been squeezed. After fixing they are white in color, leathery in texture, and the stigma along which ovulation occurs has a smooth margin, never having a torn appearance (Fig. 6).

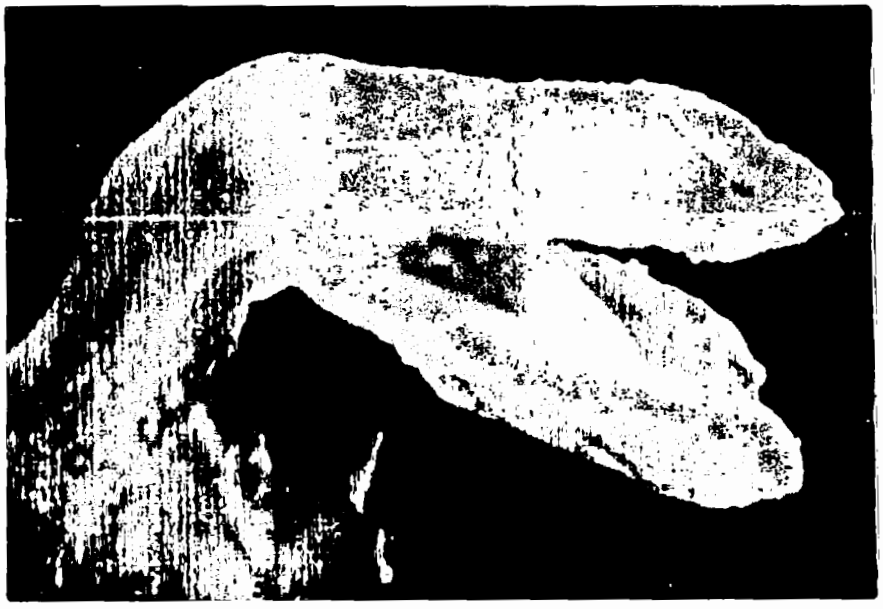

Figure 6. Large follicle just after ovulation.

Figure 7. Two follicles in an advanced stage of regression; the characteristic wrinkled appearance and "dimple-like" indentation are very conspicuous on the upper follicle.

As regression proceeds into the final or third stage, the follicle becomes smaller. Approximately 5 weeks after ovulation, follicles are reduced in size to 1 - 3 mm. in diameter (Figs. 7 and 8). Although follicles are very small at this stage, they can be easily counted because of their distinctive shape and color. They are red to reddish-orange or brown, and have an unmistakable wrinkled appearance. Older follicles of this group take on a circular shape and have a dimple-like indentation at their center (Fig. 7). This indentation is believed to be the remains of the opening through which the ovum passed.

Follicles from the second stage of regression fall between the two stages described above and are difficult to identify. Atrophy has advanced far enough to obscure many features present in the first stage of regression and they are much smaller. They are not as small as follicles in the last or third stage of regression, but are not as well pigmented making them harder to distinguish (Fig. 8). These follicles are also more flimsy than those from the other two stages of regression, making them more susceptible to damage. Counts obtained from ovaries having a large proportion of follicles from this stage could be expected to have more variation than counts of ovaries having follicles mainly from the third stage.

Analysis of data to determine what effects the different stages of regression have on follicle counts supports this hypothesis. Counts from ovaries having a long regression time

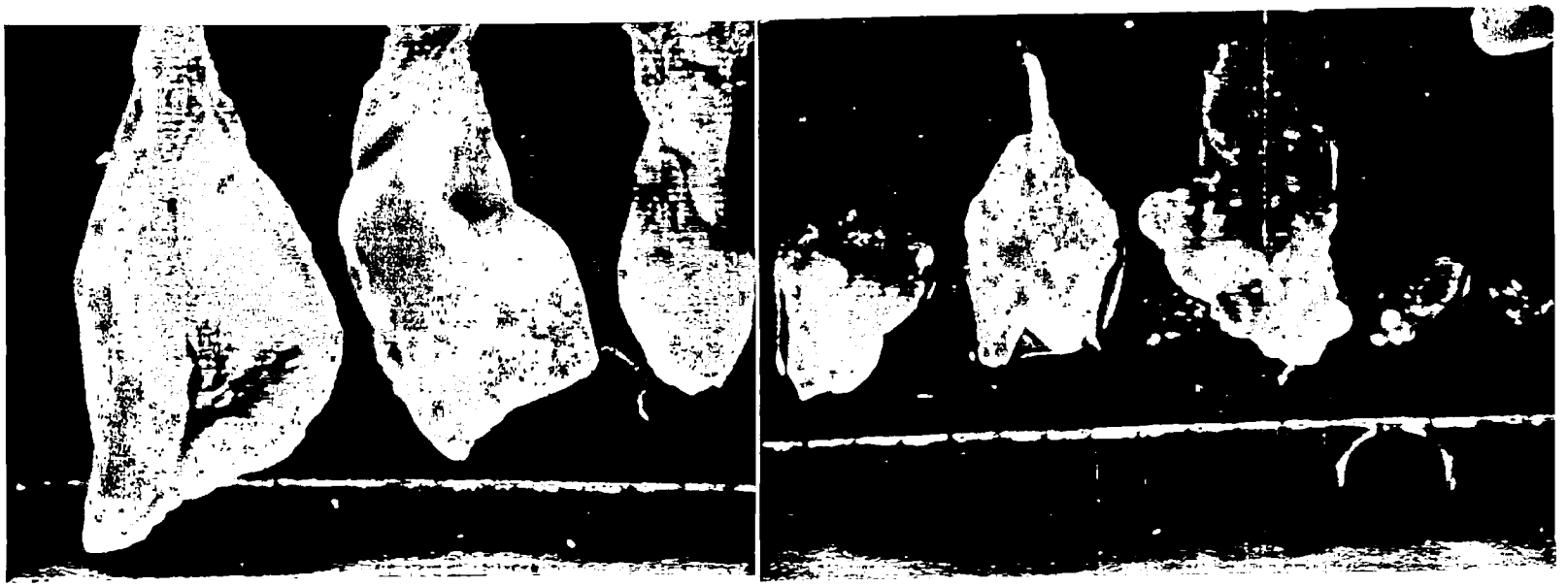

Figure 8. A series of atretic follicles showing the different stages of atresia. Follicles in the middle of this series are those most responsible for questionable follicles.

were not significantly different from the known laying record, ($t = 0.356$, $P > 0.05$), but for counts from ovaries with short regression periods, a significant difference was detected ($t = 2.732$, $P < 0.05$).

Concern for the amount of variance contributed by follicles in the second stage of regression was the reason for considering the time between the last egg and death as the regression period. The hypothesis was that these atretic follicles contributed more variance to counts than the third-stage follicles. By considering regression time as the period between the last egg until death, hens could be divided into groups according to the number of critical second-stage follicles on their ovaries. It is apparent that hens laying until the time of death would have more second-stage follicles than hens that have ceased laying some time before their death. If the hypothesis is true, counts from ovaries having fewer second-stage follicles should be significantly more reliable than those having shorter regression periods.

Results of follicle counts expressed as deviations from the laying record for four groups of ovaries having different regression periods are shown in Table 1. The value for F indicates there was not a significant difference between the counts at the 0.10 level (Table 2).

The difference between follicles counted and eggs laid for each hen was used, rather than the raw data, for computing F.

Table I. Deviation of Follicle Counts from the Known Laying Record of Hens Having Four Different Regression Periods.

	Group 1 0-10*	Group 2 15-60	Group 3 65-100	Group 4 100-125
	46	-13	7	14
	20	-2	-8	-18
	-2	7	-4	1
	2	-7	0	-6
	3	5	-2	24
	50	21	-2	-17
	49	2	4	5
	3	24	-2	41
	25	16	-2	-14
	2	1	11	-1
	1	-16	-15	7
	0	2	17	-7
$\bar{x}$ =	12.083	3.33	.33	2.41

* Days between laying of last egg and death of hen.

Table 2. Analysis of Variance and Orthogonal Comparisons of Follicle Counts from Hens Having Different Regression Periods.

Source of Variation	d.f.	Sum of Squares	Mean Squares	F
Between regression periods	3	966.75	322.22	1,165 N.S.
Error	44	12,165.17	276.48	
Total	47	13,131.92		

Comparisons	S.S.	F
1vs2,3,4	910.028	$\frac{910.028}{276.48} = 3.291^*$
3vs2,4	51.681	$\frac{51.681}{276.48} =$ N.S.
2vs4	5.042	$\frac{5.042}{276.48} =$ N.S.

* Significant for 1 and 44 d.f. at 0.10 level.

This was necessary because of the large variation in the number of eggs laid. Any significant F value calculated from the raw data could have been interpreted as a significant difference between the number of eggs laid by different hens. A significant F calculated from deviations of follicles counted from known eggs laid would, however, be an expression of the variation existing between counts made from different groups.

Results of the orthogonal comparisons show that only group 1 was significantly different from the rest. Group 3 was not significantly different from groups 2 and 4, nor group 2 from group 4 (Table 2). Because the counts from group 1 deviate the most from the egg laying record; (12.083 follicles per count on the average), the counts from the other groups are significantly more reliable than those of group 1. Since group 1 has the shortest regression period, it will have the most follicles in the second stage of regression. This offers statistical evidence that follicles in the second stage of regression contribute a significant amount of variation to the counts. Group 2 will have only a few follicles remaining in the second stage and the last two groups will consist entirely of follicles in the third stage of regression. The increase in deviation of follicles from the laying record of group 4 over group 3 (from .333 to 2.41) may be due to the final resorption or fading of the old third-stage follicles. Buss et al. (1951) noted follicles

from hens that laid during regular breeding season were beyond recognition by February.

Effects of Freezing

Only data including questionable follicles were used in making comparison between frozen and unfrozen material. Follicles counted and eggs laid were compared for unfrozen ovaries, and no significant difference was found ($t = 1.217$, $P > 0.05$). The same comparison made for frozen ovaries also indicated no significant difference ($t = 1.651$, $P > 0.05$). It is apparent that freezing of ovaries does not adversely affect atretic follicle counts. The correlation coefficient obtained between eggs laid and follicles counted for frozen material was 0.8869 as opposed to 0.7587 for unfrozen material.

Color Reactions

Attempts to utilize strong acids in producing color change reactions with the carotenoid pigment and related compounds present in ovulated atretic follicles were unsuccessful. The Schultz method for determining cholesterol did produce a blue-green color reaction in atretic follicles, but the acid solution used in this technique was too harsh and caused deterioration of ovarian tissue. Washing the acids off after shorter periods of exposure was attempted, but the color faded after rinsing.

Perchloric acid produced a color reaction similar to that described above in some atretic follicles, however, others remained the same color as before. Also, the reaction was observed in several follicles that were believed to be unovulated.

Concentrated sulphuric acid was found too harsh and damaging to the ovarian tissue to be of use. Diluted concentrations of this acid did not produce color reactions. Hydrochloric acid was also used but it did not react.

Staining Techniques

In initial experiments, attempts to properly destain tissue that had been stained with Sudan Black B were unsuccessful. Only a small amount of this stain was available and a suitable destaining agent had not yet been found so it was not tested extensively. When it was found that other stains would produce suitable results, work on Sudan Black B was abandoned. This stain may be capable of suitable results and further work is needed.

Sudan III, Sudan IV, and Oil Red O were found to selectively stain ovulated atretic follicles. This was first noticed after tissue bulk stained by these substances were placed in FAA for storage. Leaving the tissue in the FAA for several days thoroughly destained non-follicular ovarian tissue, leaving it with a slight pinkish cast but approximately the same color as before staining.

However, the stain was not removed from the atretic follicles (Fig. 9).

Need for a better destaining agent was soon apparent as FAA was found too slow and inefficient. Concentrated solutions of acetone, isopropyl alcohol, and ethyl alcohol were not usable as destaining agents. Acetone completely destained all tissue including atretic follicles. Isopropyl alcohol left atretic follicles only slightly darker than other tissue. Tissue left in pure ethyl or methyl alcohol for one week failed to destain. Acid ethyl alcohol also did not destain tissue. A solution of acetone and an equal part of water destained the tissue too much. A 1 : 1 solution of isopropyl alcohol and water was useful in producing the desired destaining effects. Atretic follicles were stained while non-follicular tissue was thoroughly destained. Time required for destaining varied depending on the individual ovary and the degree to which it was stained. A minimum of 48 hours was usually required to properly destain material, but some ovaries took up to twice this long.

The optimum time period for staining tissue was found to be one hour. Material stained for longer periods eventually destained, but took much longer to do so. Also, additional contact with stain did not result in additional benefit in staining of atretic follicles.

Figure 9. Stained atretic follicles on ovarian tissue. (Note that other tissue was left well destained.)

Oil Red O was used to stain follicles for determining reliability of technique. Comparison of sample means obtained from follicle counts of 23 ovaries, before and after staining, was highly significant at the 0.05 level ($t = 3.111$). Counts made from stained material are more reliable than those made from unstained material. For example, counts made from unstained material deviate, on the average, 6.130 follicles each from the known laying record, while each count from stained ovaries varied only -0.261, on the average, from the known eggs laid (Table 3). Since follicles counted were subtracted from eggs laid, a positive deviation would indicate fewer follicles were counted than eggs laid. Therefore, follicles counted for each ovary of unstained material were, on the average, a little over six follicles less than needed to account for the eggs laid.

The greater variations between knowns and observed counts made from unstained and stained material are illustrated in Figures 10 and 11, respectively. For stained material, points of the scatter diagram cluster about the regression line more than they do for unstained material. More narrow confidence limits placed around the regression line of the stained material is evidence of improved accuracy with this technique.

The staining technique developed in this study greatly helped in identifying ovulated atretic follicles, but interpretation of the material was still required to the same degree as for unstained material. It is recommended that

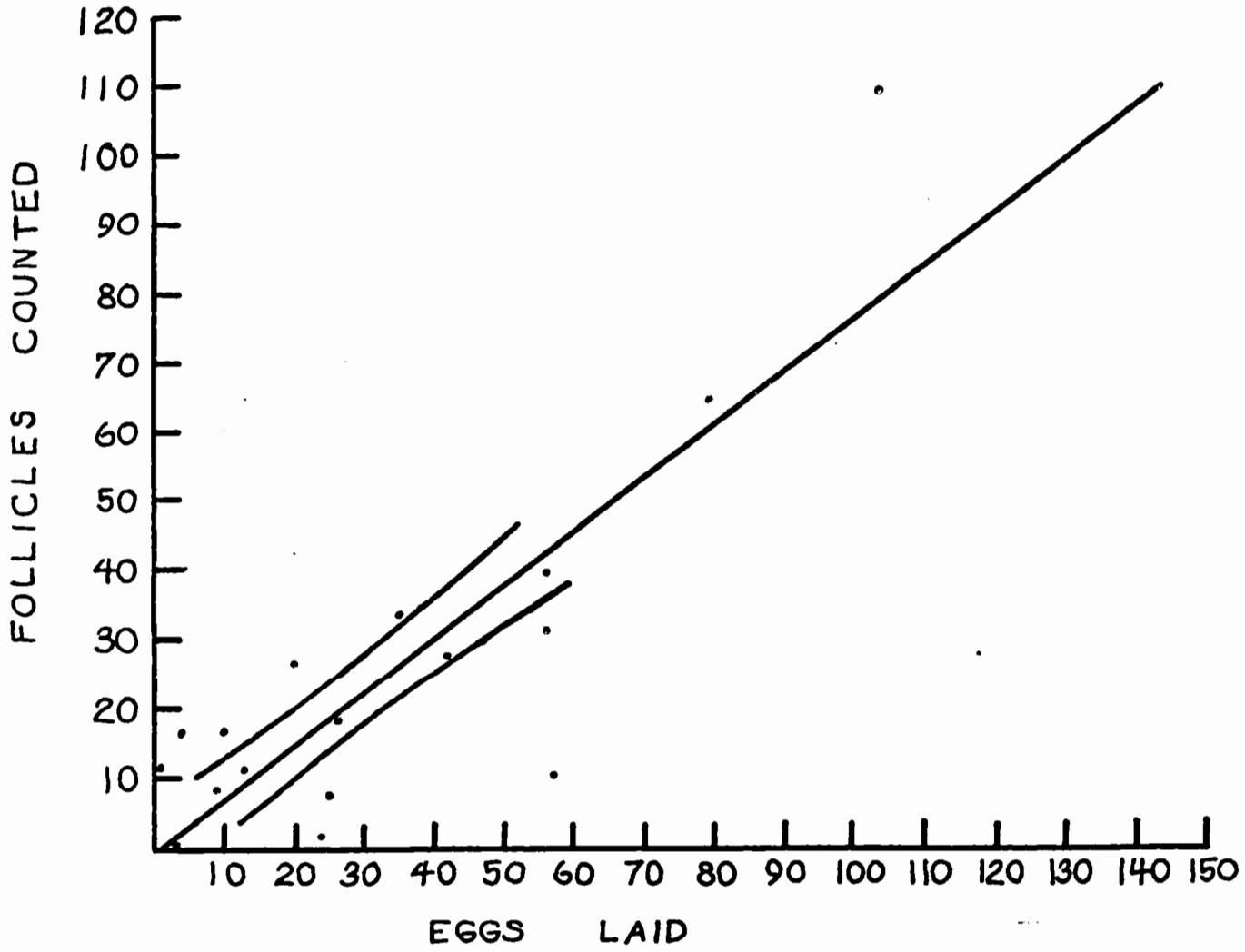

Figure 10. Relationship between follicles counted and eggs laid for unstained ovaries (confidence belts calculated for $P > 0.05$).

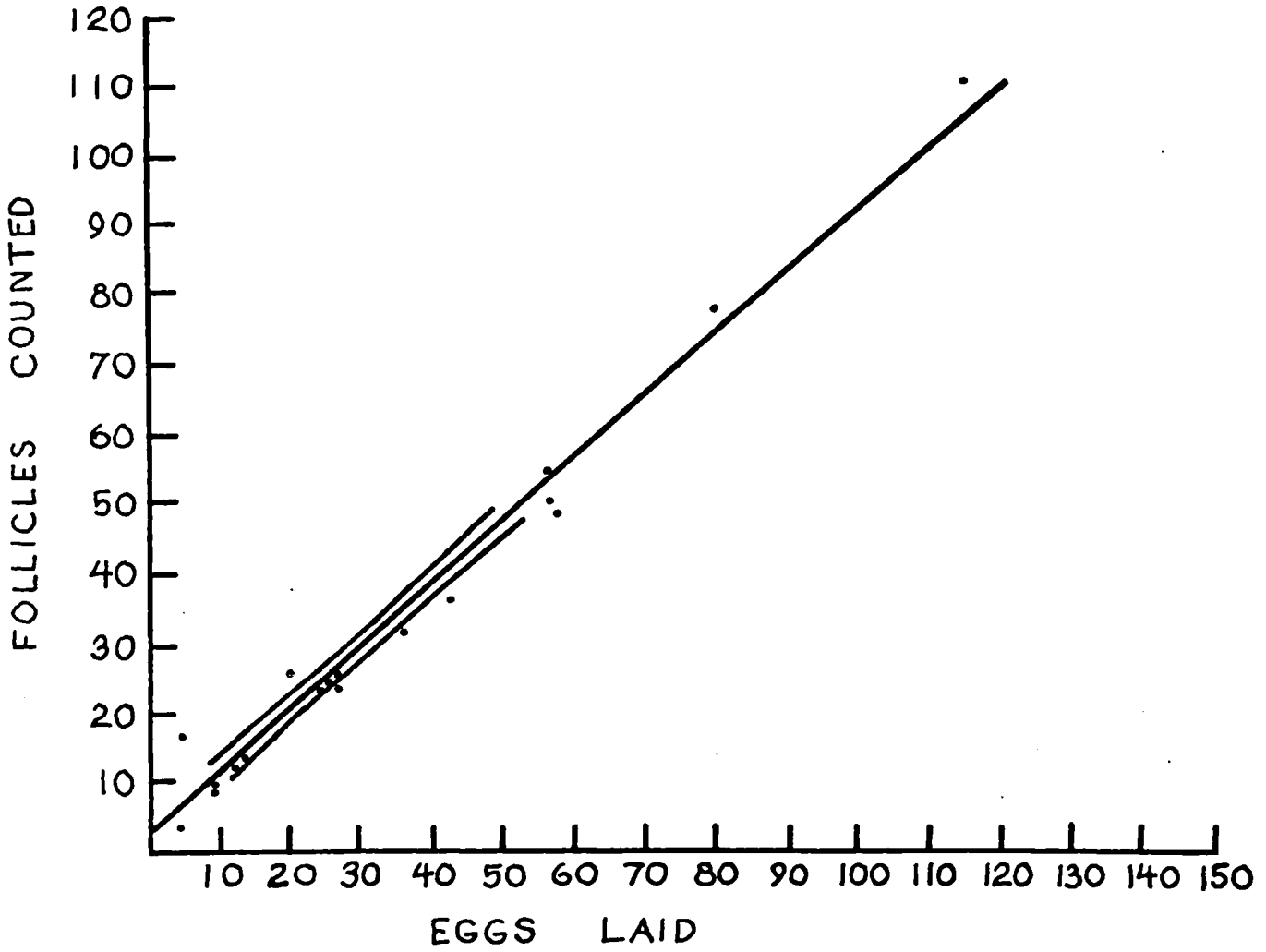

Figure 11. Relationship between follicles counted and eggs laid for stained ovaries (confidence belts calculated for $P > 0.05$).

before counts are attempted from stained material that an investigator become thoroughly acquainted with the technique of counting atretic follicles from unstained ovaries. For example, fat tissue is stained just as readily as are atretic follicles (Figure 12) and often bits of yolk or other debris are also stained. Identification of these non-follicular artifacts in an unstained condition is essential before the investigator can expect to identify them after they have been stained.

The main contribution of staining was the ability to bring out features of atretic follicles, too obscure in an unstained condition to be detected. Due to this many of the questionable follicles were eliminated from the counts. Questionable follicles were reduced from 75 in unstained material to 44 in stained material (Table 3). In some ovaries, follicles that had been invisible, were easily discernible after being stained. An example was the ovary from hen number 160 which laid 25 eggs and yet only eight atretic follicles could be found on the ovary before staining. After staining, 25 atretic follicles were counted. Figure 13 shows several follicles from this ovary after being stained.

Figure 12. Stained fat tissue (upper) and atretic follicles (lower).

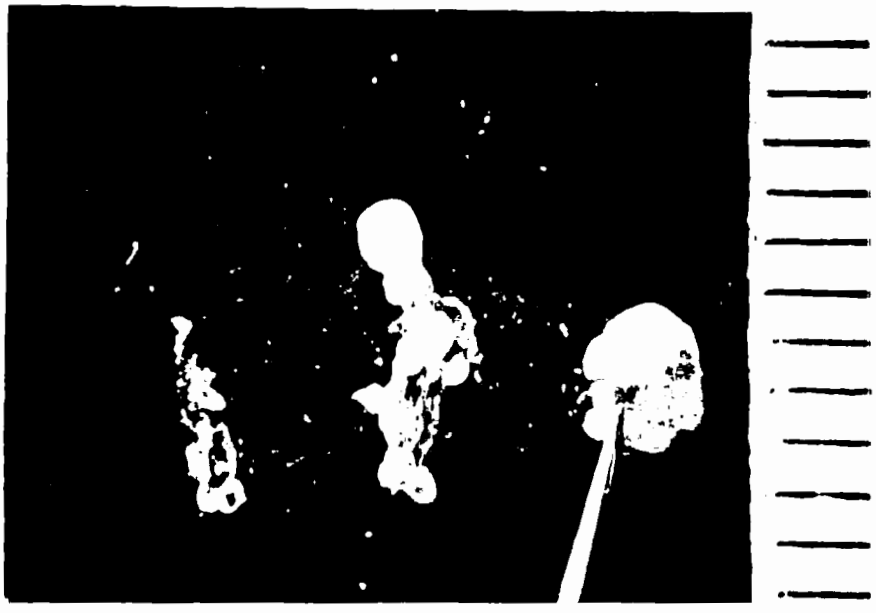

Figure 13. Extremely small and obscure follicles brought out by staining.

Table 3. Results of Follicle Counts Before and After Staining.

Hen No.	Known Eggs Laid	Unstained Follicles Counted	Unstained Deviation From Known (X_1)	Stained Follicles Counted	Stained Deviation From Known (X_2)	$X_1 - X_2$
100	42	21 + 7?[1]	14	34 + 2?	6	8
101	0	0 + 0	0	0 + 0	0	0
105	35	30 + 4	1	32 + 0	3	-2
106	57	8 + 3	46	42 + 6	9	37
107	79	56 + 9	14	73 + 4	2	12
113	4	14 + 3	-13	14 + 3	-13	0
114	56	25 + 7	24	47 + 3	6	18
117	26	18 + 1	7	26 + 0	0	7
132	20	22 + 5	-5	26 + 0	-6	1
137	0	4 + 1	-5	3 + 1	-4	-1
140	10	14 + 3	-7	11 + 1	-2	-5
150	113	99 + 11	3	101 + 9	3	0
160	25	4 + 4	17	23 + 2	0	17
170	56	39 + 1	16	50 + 4	2	14
588	13	8 + 4	1	13 + 1	-1	2
591	0	0 + 2	-2	1 + 1	-2	0
628	12	0 + 4	8	11 + 1	0	8
629	24	1 + 1	22	20 + 3	1	21
630	9	7 + 2	0	9 + 1	-1	1
631	9	0 + 0	9	8 + 1	0	9
633	1	10 + 2	-11	9 + 0	-8	-3
636	3	0 + 1	2	3 + 1	-1	3
637	0	0 + 0	0	0 + 0	0	0
Total	594	380 + 75?	141	556 + 44?	-6	147
$\bar{x}$	25.826	19.782	6.130	26.087	-.261	6.391

1. ? -- denotes questionable follicles

Variation Between Investigators

Follicle counts including questionable follicles made by five different investigators are presented in Table 4. Statistical calculations were made from deviations of follicles from known laying record for reasons discussed earlier.

No significant differences were found in analysis of variance (Table 5) of counts made by different investigators ($P > 0.05$).

The limited scope of this experiment restricts the assumptions that may be made concerning the variation between counts of different investigators. The inference that no significant difference exists between counts of individual investigators cannot be made for techniques other than the one described in this paper.

Prediction of Egg Production

Figures 14 and 15 are the regression lines for prediction of mean egg production from follicle counts of both unstained and stained material, respectively. It is hoped that these can be used by other investigators for calculating the egg production for the preceding breeding season. However, it should be kept in mind that there might be racial or geographic differences in regression times.

Table 4. Results of Follicle Counts by Five Investigators (Data Listed as Differences from Known Eggs Laid and Includes Questionable Follicles).

Hen No.	Known Eggs Laid	Investigator				
		#1	#2	#3	#4	#5
102	3	12	12	25	21	20
107	79	-17	-22	-20	11	-14
111	43	-5	-8	-5	26	10
112	35	-29	-11	1	-12	8
115	72	-28	-12	-9	-9	6
123	16	-11	-6	0	-2	5
126	26	-6	-1	-7	10	4
151	51	9	21	40	31	25
164	32	-16	-13	1	-5	8
165	18	1	0	3	-6	6

Table 5. Analysis of Variance of Counts by Different Investigators.

Source of Variation	d.f.	Sum of Squares	Mean Squares	F
Treatment	4	2,049.72	512.43	2.55 N.S.
Error	45	9,039.00	200.87	
Total	49	11,088.72		

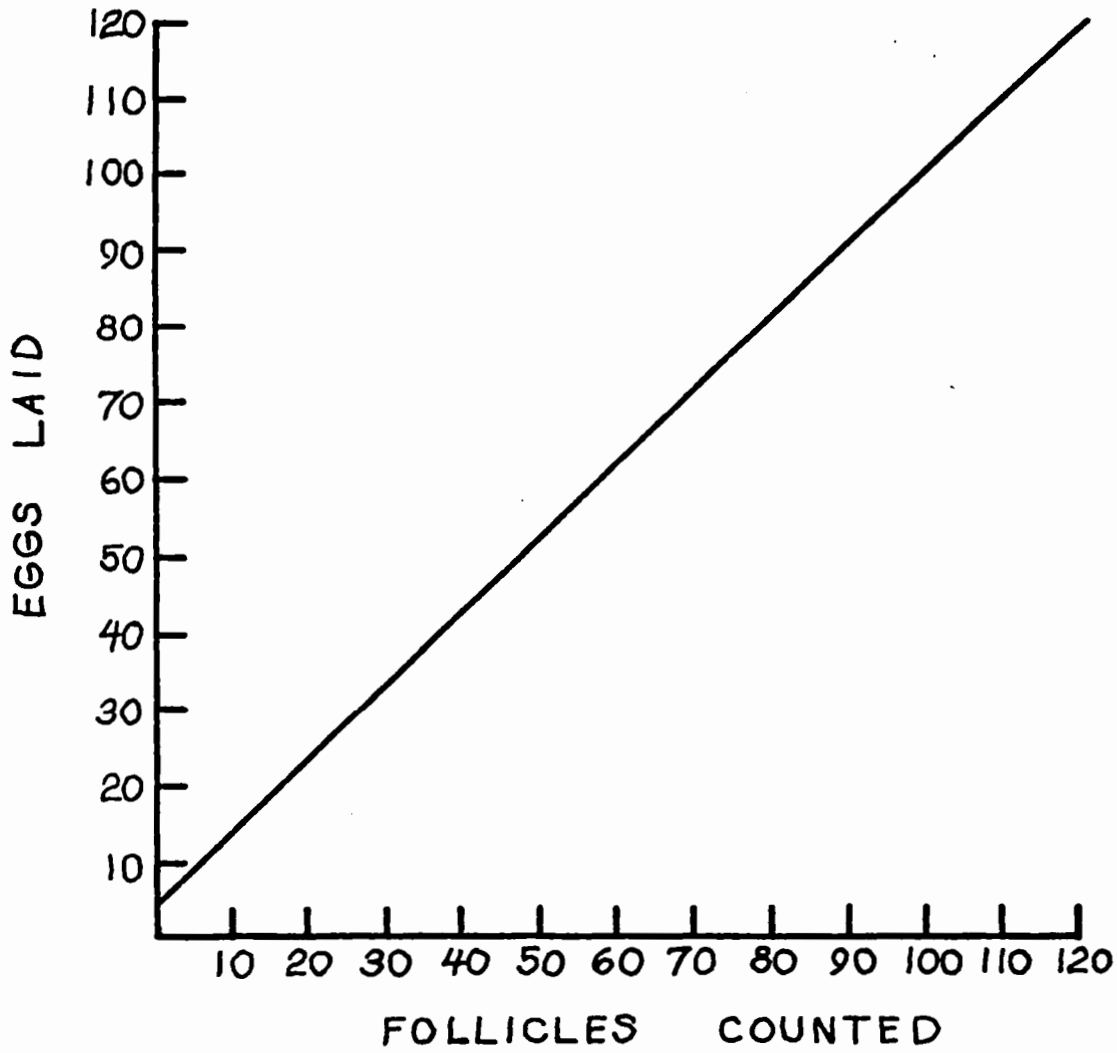

Figure 14. Regression line for prediction of mean egg production from unstained material.

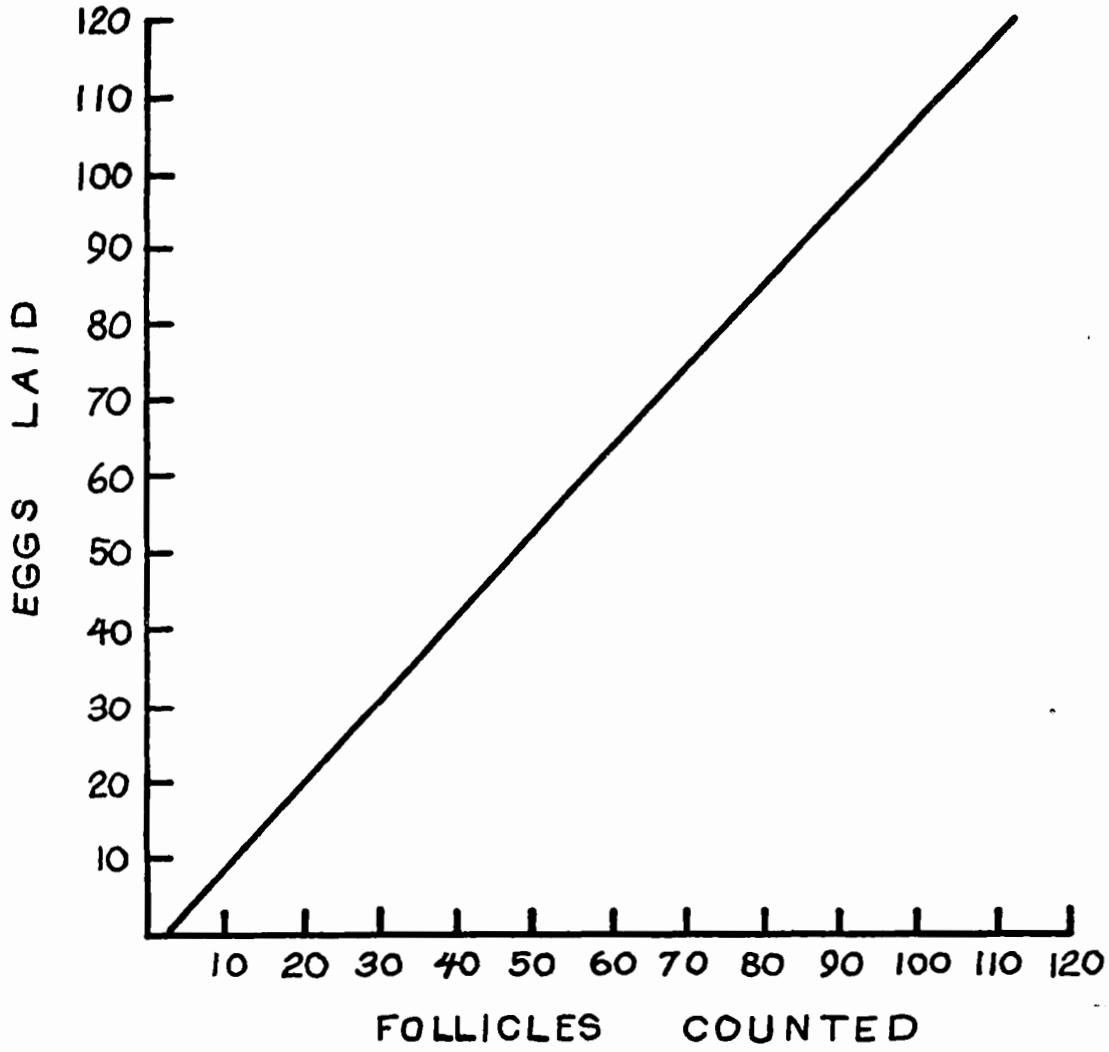

Figure 15. Regression line for prediction of mean egg production from stained material.

SUMMARY AND CONCLUSIONS

From statistical evidence presented above, and from observations made on the material used in this study it was found that questionable follicles should be considered true ovulated atretic follicles and be included in counts. Most questionable follicles were believed to originate from ovulated follicles that were more difficult to identify because of their particular stage of regression. Follicle counts from hens having more atretic follicles from this stage of regression differed significantly from counts of hens having fewer or no follicles from this stage of regression. A certain number of questionable follicles also resulted from dissection, but this number will not be significant if proper techniques are used. The extent of questionable follicles contributed by unovulated atretic follicles was not determined.

It would seem that an optimum regression period does exist from which the most accurate counts of atretic follicles can be made. Ovaries should be collected for counts at a time when it can be reasonably certain that second-stage follicles have been eliminated from the ovaries, and before the time when the atretic follicles have been resorbed beyond recognition. The evidence from the data of this study indicates that collection of wild hens for the purpose of making atretic follicle counts should be no earlier than 5 weeks nor later than 14 weeks after general cessation of laying at the end of the breeding season.

Freezing of ovaries will not affect subsequent follicle counts.

Attempts to utilize strong acids in color-change reactions with the carotenoid pigments of atretic follicles were not successful, but a selective staining technique was developed using Oil Red O, Sudan III and IV mixed with propylene glycol. These stained faded or obscure follicles so they were easily counted, and eliminated many questionable follicles from the counts. Counts made from stained material were found to be significantly more accurate than counts from unstained material when compared with known records of each hen.

It is believed accuracy of follicle counting is sufficient to justify its use for prediction of mean egg production. Lack of significant variability between follicle counts of investigators compared in this study indicates that similar results would be obtained by others if the techniques described here were used.

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Appendix. Results of Follicle Counts From Unstained, Frozen and Unfrozen Ovaries Plus Counts Obtained After Staining.

Hen No:	Known Eggs Laid	Follicles Counted			
		Unstained		Stained	
		+?	-?	+?	-?
100 f*	42	28	21	36	34
101 f	0	0	0	0	0
102	3	21	17		
105	35	34	30	32	32
106 f	57	11	8	48	42
107 f	79	65	56	77	73
110 f	42	22	18		
111	44	53	49		
112	36	42	37		
113 f	4	17	14	17	14
114 f	56	32	25	50	47
115	73	75	66		
116	91	89	83		
117 f	26	19	18	26	26
119	41	49	46		
121 f	20	28	24		
122 f	7	9	7		
123 f	17	21	17		
126 f	29	29	24		
127 f	5	20	17		
128	6	23	18		
129	22	17	12		
130 f	3	5	4		
131 f	51	44	36		
132 f	20	27	22	26	26
133	24	19	15		
134	75	34	28		
136	0	0	0		
137 f	0	5	4	4	3
140 f	10	17	14	12	11
141	62	41	33		
142	18	15	10		
143	73	23	18		
144	16	30	28		
145	94	46	38		
146	51	2	1		
147	4	13	9		

Appendix (Cont'd.).

Hen No.	Known Eggs Laid	Follicles Counted			
		Unstained		Stained	
		+?	-?	+?	-?
148	21	22	20		
150 f	113	110	99	110	101
151	52	77	68		
152 f	24	26	21		
153	48	46	40		
154 f	21	17	15		
155 f	2	4	3		
156	9	8	7		
157 f	4	6	5		
160 f	25	8	4	25	23
161	1	0	0		
162	24	17	13		
163	38	14	10		
164	32	39	32		
165	18	21	19		
166	0	14	11		
167	1	0	0		
170 f	56	40	39	54	50
171 f	0	0	0		
172 f	5	4	3		
173	0	0	0		
174	17	33	23		
175	10	11	10		
176	0	2	0		
177 f	36	30	23		
588	13	12	8	14	13
591	0	2	0	2	1
628	12	4	0	12	11
629	24	2	1	23	20
630	9	9	7	10	9
631	9	0	0	9	8
633	1	12	10	9	9
636	3	1	0	4	3
637	0	0	0	0	0

*f = Frozen