THE PHYSIOLOGY OF YOLK FORMATION, ESPECIALLY THE

VITELLINE MEMBRANE AND THE MECHANISM OF

OVULATION IN THE FOWL

By

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INTRODUCTION

Although many phases of the development of the egg yolk of the domestic fowl have been studied in complete detail in some aspects, there are still a number of important points that need further clarification. The problems to be considered in this thesis are three:

1. Rate of yolk secretion by the fowl during the rapid growth of the follicle

2. The origin and chemical composition of the vitelline membrane

3. Environmental factors which may induce ovulation.

The egg of the domestic fowl is produced for the greater part in the seven to eleven day period before laying. The ovum at the beginning of this period is a small beadlike body on the ovary seven to nine mm. in diameter, weighing about one-fourth gram. During the next six to ten days it grows rapidly until when it is shed from the ovary it is 25-35 mm. in diameter and weighs from ten to twenty grams. The oviduct secretes the albumen, membranes and shell around the yolk during the last 24 to 30 hours before the egg is laid, producing a complete egg of fifty to sixty grams.

The formation of the mature yolk and ovulation are of fundamental importance in the egg production of the fowl and are evidently quite complex in their physiological mechanism. The formation of the albumen, membranes and shell is in contrast a relatively simple mechanical reflex reaction according to Pearl (28). Any small body passing through the oviduct

stimulates the secretion of albumen, membranes, shell and laying in normal sequence.

The growth of the ovum of the fowl has been studied by many workers. Marza & Marza (24) have reviewed the subject from a histological and histochemical point of view. Conrad & Scott (7) recently have reviewed the work on the complete formation of the egg and its parts.

REVIEW OF LITERATURE

The development of the ovum. The development of the primordial germ cell into the mature ovum has been described in several publications. The authors have been able to identify the primordial germ cells during the early embryonic cell divisions and to follow them in the fowl. Swift (43), Firket (13) and Goldsmith (16) found the primordial germ cells anterior and anterolateral to the primitive streak in the embryo after twelve hours of incubation. These cells migrate into the mesoderm when it is formed and with the development of the vascular system are transported in the blood to the germinal ridges. As the germinal ridge develops into the stroma of the ovary and the germinal epithelium forms the sexual cords of the first and second proliferation the germ cells migrate outward, finally coming to rest in the cortex of the ovary.

Goldsmith (16) found a slight degeneration which he considered synapsis on the fifteenth day of incubation. The cells soon recover their original size and are now primary ova.

The primary ova undergo a lengthy development up to the maturation period just before ovulation. As has been pointed out by Marza & Marza (24), during this time the ovum grows from 15 to 20 microns on the twentieth day of incubation to 2.5 to 3.5 centimeters in the mature ovum. This is a growth of approximately 2,000 times in diameter.

In the two-day-old chick, follicular cells derived from the germinal epithelium are beginning to form around the ova as the first indication of the follicular ring. At 5 days they are definitely enclosed by the follicular cells according to Holl (18). The earlier stages of ova are often called the extra-follicular stage, and, after the formation of the follicles, the intra-follicular stage.

According to the many works on nuclear changes, they are intensified during the extra-follicular period and at the end of the intra-follicular period. During the rest of the intra-follicular period nuclear changes are very slight.

The nucleus is centered during the earlier extra-follicular stages. Shortly after the beginning of the intra-follicular stage the nucleus moves to the surface and comes to lie close to the vitelline membrane. The nucleus lies in a thickening of the peripheral layer of the protoplasm known as the germinal disc. As the germinal disc increases in extent and thickness, an inflow of protoplasm into the disc must take place. The nucleus becomes elliptical and later the outer surface is flattened against the rounded vitelline membrane, the inner surface remaining convex. Bartelmez (2)

found that the point to which the nucleus migrates is away from the surface of the ovary, and the nucleus comes to lie ajacent to the pedicle of the follicle when the latter projects from the surface of the ovary.

The nucleus increases in size with the growth of the ovum; in the youngest ovum its diameter is about 9 microns, in the ripe ovum it is flattened and may measure, according to Sonnenbrodt (40), 455 microns in diameter and 72 microns in thickness. Brambell (5) found that the nucleoplasmic ratio decreases according to the development of the ovum. The nucleus is two-thirds of the cell in ova of 30 to 50 microns in diameter and only one-half of the cell in ova 50 to 70 microns in diameter. A further decrease in this ratio takes place as the ovum grows, especially during the last rapid growth period.

Growth of the ovum. The growth of the ovum is not uniform after its formation. While changes in rate of growth are known to occur, the growth rate and length of time in the various growing phases are not well known. Certain morphological characteristics are found to be correlated with the ova of definite sizes, but the length of time these characteristics are present is known only through changes that occur when the ova are larger in size. Periods of rest are thought to occur at certain sizes.

A long slow growth period of the follicle up to about 6mm. in diameter followed by a short rapid growth period has been observed. The growth rate during the first part of the

slow growth phase is not well known. A number of the fol-
licles appear to grow after hatching until they are 80 microns
in diameter. Brambell (5) found that a number of follicles
stopped at this size and did not continue growth for several
weeks or more.

Of the growth rate from 80 microns to approximately 3
mm. in diameter very little is known. After pediculation
when the follicle may be measured more easily in the fresh
state the growth rate is better known. Romanoff's (38)
measurements, taken of all the follicles found on the ovary
of a hen laying at a high rate of egg production, show the
changes in growth rate after pediculation very well. These
data are given in Table I and are shown partially plotted in
Chart I.

A slow growth period from when the follicle pediculates
at 12 milligrams up to 128 milligrams may be observed. This
would appear to cover 13 days and give an average rate of
yolk deposition of approximately nine milligrams per day.
The irregularities noted in the plotted data for this period
might be due to irregularities of yolk formation, or a be-
ginning of segregation of follicles into groups that would
form a single clutch of eggs. Riddle (31) found that the
follicles from 3.0 mm. to 6.0 mm. in diameter grew only one
twenty-fifth as fast as the follicles during the later rapid
growth period.

Table I. Measurement of ova on ovary of hens in high rate
of production

Series of ova	Growth of fowl's ovum				Ratio H/W
	Average weight (in grams)	Percentage dry matter	Height (in mm.)	Width (in mm.)	
After laying	17.650	52.36	14.5	39.0	.37
Before laying	17.076	52.76	16.0	35.5	.45
1	15.029	54.54	23.5	32.5	.72
2	10.782	53.20	20.5	29.5	.69
3	5.572	51.05	17.0	23.5	.72
4	2.287	48.15	12.0	17.5	.71
5	.608	39.30	7.5	10.5	.71
6	.250	18.57	6.0	8.5	.73
7	.199	17.60	5.5	7.5	.75
8	.157	15.18	5.25	7.0	.77
9	.144	14.45	5.0	6.5	.78
10	.128	14.25	4.25	5.5	.81
11	.122	14.12	3.5	4.5	.80
12	.115	13.47	3.25	4.0	.86
13	.095	11.90	3.0	3.75	.86
14	.089	14.43	3.0	3.5	.86
15	.083	13.58	3.0	3.5	.86
16	.071	11.32	3.0	3.5	.86
17	.065	15.31	3.0	3.5	.86
18	.063	13.31	3.0	3.5	.86
19	.058	12.80	3.0	3.5	.86
20	.045	--	3.0	3.5	.86
21	.042	--	3.0	3.5	.86
22	.035	--	2.75	3.25	.85
23	.016	--	2.75	3.0	.92
24	.013	--	2.75	3.0	.92
25	.012	--	2.5	2.75	.91
26	.012	--	2.5	2.75	.91
27	.012	--	2.5	2.75	.91
28	.012	--	2.5	2.75	.91

From Romanoff (38). Biochem. Jour. 25, 994 (1931).

CHART II

Weight of Follicles Found in
Ovary of Hen in Rapid Rate of
Egg Production
(Plotted from Data by Romanoff)

Fol.
Wt.
in
Gms.
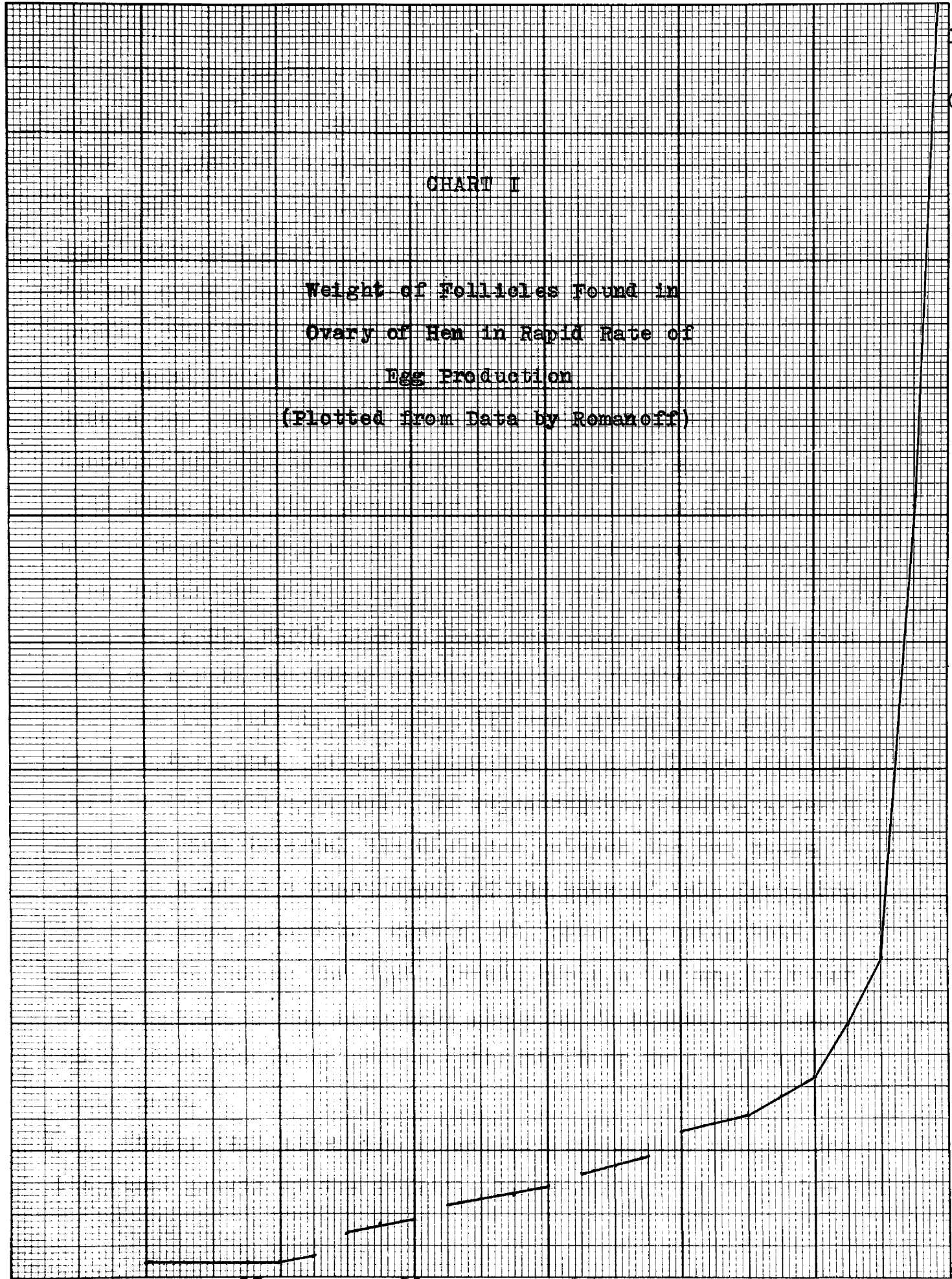
Series of Follicles by Decreasing Weight

The plotted data also show an increased growth rate
after the follicle has passed 128 milligrams in weight.
From the plotted data this would appear to be a single growth
cycle up to the completed mature follicle, beginning slowly
and increasing rapidly. Riddle (32), who has studied this
latter rapid growth period, separates the cycle into two
parts. The first part is from 6 mm. to 9 mm. in diameter and
the second from 9 mm. in diameter up to the mature follicle.
Riddle (32 found a daily increase of from 0.25 to 0.5 mm. in
diameter per day in the growth from 6-10 mm. Marza & Marza
(24) state that this phase may last from one to five days or
may be completely lacking. Riddle (32) observed stratifica-
tion within the yolk material during this phase.

The final rapid growth phase has been studied more than
that of any of the other growth cycles. Riddle (31) found
that the fat soluble dye Sudan III was transmitted into the
yolk of the ovum. After feeding the dye a definite ring was
left in the yolk material. By repeating the feeding after a
definite time interval the distance between the two rings
could be used as a measurement of the rate of yolk formation
for that time interval. Riddle (31) found by using this
method that during the final growth cycle a two mm. increase
in the radius of the ovum took place in 24 hours. He also
states that the rapid growth cycle may last from five to
eight days.

Stieve (42) attempted to measure the growth rate during
the final growth of the follicle by measuring the follicles

of hens that were in a definite rate of egg production. He thought that the difference in size between the follicles would give an indication of the daily growth rate. In general he confirmed Riddle (31) on the rate of growth during the final rapid growth period. He concluded, however, that the rate of yolk deposition during this final rapid growth period was quite variable. Follicles might grow rather slowly one day and quite rapidly the next.

Warren & Conrad (47) have recently studied the growth of the ovum during the final rapid growth stage. They injected a colloidal suspension of Sudan III intravenously at 24-hour intervals and calculated the radius increments and increase in weight from the distances measured between the rings formed in the yolk. By the injection of the dye sharper edges were found on the rings so that measurements could be made more accurately. By making injections at 12-hour intervals no difference in the rate within the daily cycle could be observed.

From the ninth to the fourth day before ovulation the rate of yolk growth was found to increase, with a decrease in the rate after the third day. Some growth could be detected even within an hour of ovulation.

A difference in growth rate of the ova was not observed, for different ova from the same hens or of different hens. They state that differences in mature yolk size might be attributed to length of the period of growth rather than to the difference in rate of growth. Differences in rate of

egg production did not seem to influence the rate of growth
of the individual follicles.

Phases of yolk formation. The growth of the ovum is
due primarily to the rate of deposition of yolk. The yolk
globules deposited are found to differ histologically as the
ovum grows. The appearance of the different layers of fat
globules and vacuoles formed during the slow growth period
has been described by Van Durme (45). In this study it was
found that layers of fat globules and vacuoles were deposited
in definitely arranged formations in the ooplasm of ova of
various sizes. Three phases of yolk formation are distin-
guished.

In the small ovum of about 100 microns in diameter the
vitelline body or yolk nucleus of Balbiani may be observed.
An accumulation of mitochrondria and Golgi elements have
been observed at the periphery of the body by Van Durme (45)
and Brambell (5). When the ovum has reached 200 microns in
size the body of Balbiani begins to disintegrate. When the
ovum is from 300 to 1,000 microns in diameter a layer of fat
globules was observed around the periphery. This layer be-
comes only dimly visible in ova of 1 to 2 mm. in diameter
(Marza & Marza, 24). This completes the first phase of yolk
formation as classified by Van Durme (45).

The second phase beginning in ova of 2 mm. diameter
consists of transparent vacuoles in the center of the ovum
surrounded by a granular cortical layer. Round yolk globules
are formed in the vacuoles after the ovum is about 3 mm. in

diameter. Marza & Marza (24) call the round yolk globules
formed in this manner primordial yolk to distinguish it from
the yolk formed later. Yolk is not formed within the ovum
until after pediculation of the follicle. This fact has not
been pointed out previously but the author thinks it is a
point to be considered in the development of the ovum. In
the further growth of the ovum from 3.0 to 6.0 mm. in diameter
the cortical granular layer with the central portion of pri-
mordial yolk seems to continue.

With the beginning of the rapid growth period in ova of
from 6 to 9 mm. in diameter layers of primordial yolk mixed
with white yolk globules are laid down, according to Marza &
Marza (24). They state that the white yolk globules formed
at this time are different from the primordial yolk and the
true yellow yolk that is to be formed later.

At the beginning of the final rapid growth period as
distinguished by Riddle (32), large yolk globules similar to
the yellow yolk are formed which seem to have inclusions
like the white yolk globules. These have been observed and
called by Marza & Marza (24) transitional yolk. The yellow
yolk formed during the last period makes up the bulk of the
completed yolk and is responsible for the increase in diameter
from 10 to approximately 35 mm. in diameter.

The changes in appearance of the ooplasm with the changes
in diameter have been tabulated and are given in modified form
in Table II (from Marza & Marza, 24).

Table II. The relation between the diameter of ovules and
the phases of yolk-formation of the hen's egg

Phases of yolk-formation	Appearance of the Ooplasm	Diameter of the Ovules (in mm.)
First Phase:	Balbiani body	0.05-0.2
	Disaggregation of Balbiani body	0.2-0.3
	Fat cortical layer	0.3-1.0
	Dispersion of fat-globules; cortical, vacuolar layer	1.0-2.0
Second Phase:	Cortical granular layer; central vacuolar layer	2.0-3.0
	Cortical granular layer; primordial yolk (intravacuolar) layer	3.0-6.0
Third Phase:	White yolk-layers; primordial yolk-layers; yolk of transition	6.0-9.0
	White cortical yolk; yellow yolk; latebra; nucleus of Pander	10.0-35.0

From Marza & Marza (24). Quart. Jour. Micros. Sci., 78, 1935.

Marza & Marza (24) point out that the latebra found in
the mature ovum is about 6 mm. in diameter, that of the ovum
at the beginning of the rapid growth cycle. They also find
that the yolk layers in the latebra are similar to those
found in the 6 mm. ovum and conclude that the latebra con-
sists of the ovum at this time unchanged and surrounded by
the mass of yellow yolk that is formed later. No one has
attempted to explain the mechanism by which the neck of the
latebra is formed leading to the nucleus of Pander.

Some speculation has been made as to the similarity of
the different parts of the hen's egg with that of the eggs

of different animals. Marza & Marza (24) consider that the ovum up to 2 mm. has the typical appearance of the alecithal ova. The ovum of 6 mm. in diameter, like the latebra in the mature yolk, was considered by Spohn & Riddle (41) to be similar to that of the holoblastic ova of the amphibia. The final rapid growth stage is only found in the ova of the cartilagenous fishes, reptiles and birds having meroblastic cleavage.

The pigment of the yolk. The literature on the coloring matter of the egg yolk has been reviewed by Mattikow (25). Palmer (26) found that the bulk of the normally occurring pigment was xanthophyll derived from the feed. Zeaxanthin is also found and Winton & Winton (51) state that the ratio of xanthophyll to zeaxanthin is approximately 7:3. Gillam & Heilbron (15) found that cryptoxanthin may be present depending upon the diet. Capxanthin, from pimiento peppers, was also found by Brown (6) to color the egg yolk.

Henderson & Wilcke (17) found that a hen was not able to withdraw Sudan III from the colored body fat to color the yolk. This is of practical interest since it is thought that the rate of egg production was responsible for the withdrawal of the pigments from the beak, shanks and skin observed in hens when laying at a high rate of production.

Stratification in the yolk. Thompson (44) in 1859 made a diagram of the egg which has become classic in which in the yolk, stratification of white and yellow layers are shown about the latebra. Some observers have been able to find these layers while others doubt their existence. The

difference was due to the presence or absence of the yellow coloring matter, according to Balbiani (3). Wasserman (49), while unable to observe stratification, found layers of globules of different sizes. Riddle (31) thought that a daily rhythm of nutrition and blood pressure was responsible for the yellow and white yolk stratification, yellow yolk being formed during the period of high blood pressure and white yolk in the nocturnal period of low blood pressure. Riddle (31) states that the layer of white yolk of the hen's egg represents the result of yolk formation under suboptimal conditions. Gage & Gage (14) found stratification of color in hens fed with Sudan III mixed in the feed.

Conrad & Warren (8) were unable to find stratification in eggs from hens fed a uniform mash, but did find them in some farm eggs, and could produce them by various dietary regimes. They were able to produce eggs showing these alternate layers simply by restricting the feeding period to six hours per day or by feeding a mash poor in the egg coloring xanthophylls supplemented by a small amount of yellow corn or green grass each day.

Follicular membranes. The ovum and the cells around it are called the follicle. The follicle itself shows definite cellular structure. The cells which are in direct contact with the ovum are the follicular epithelial cells. D'Hollander (12) concludes that they are derived from the germinal epithelium.

The cells of the follicular epithelium do the work of

an ultra-filter of which permeability changes several times during the growth of the ovum, according to Marza & Marza (24).

In a general way variations in the depth of the follicular cells have been shown for the hen's egg by Loyez (23) and Brambell (5). Follicular cells are shown that increase in depth and in number during the slow phase of follicle growth. Loyez (23) and Brambell (5) thought that just before pediculation the follicular epithelium reaches a maximum depth and that it even may become pluristratified, and that toward the end of the ovum's growth the depth of the follicular epithelium decreases greatly. Marza & Marza (24) found that in the slow growth phase of yolk formation the follicular epithelium cells increased in depth continually. The cells are very flat at the beginning and become very deep at the end of the slow growth period of the ovum, becoming as much as 19 microns thick. During the time the follicle is from 1.0 to 3 mm. in diameter the cells maintain their depth and after the follicle pediculates the cells start to diminish in height. During the rapid period of growth the depth continues to decrease and at the end the cells are not more than 3.6 microns deep. An inverse ratio exists between yolk formation and the depth of the cells. Marza & Marza (24) considers that the cells are never stratified but psuedostratified and nuclei could be observed at two different levels in the single layer of cells.

Table III. Depth of follicular epithelial cells during
the three phases of yolk formation

Diameter of ovum (mm.)	Depth in microns	Diameter of ovum (mm.)	Depth in microns
0.03	3.8	1.5	19.0
.05	4.2	2.0	17.1
.10	5.6	2.5	19.7
.15	8.1	3.0	17.8
.20	9.0	4.0	17.1
.25	9.3	5.0	10.3
.30	9.6	6.0	7.1
.35	11.7	7.0	6.9
.40	12.2	8.7	7.0
.60	12.0	9.0	5.9
.70	14.7	10.0	5.1
.80	16.7	20.0	4.1
.90	18.5	25.0	3.6
1.00	19.3		

Modified from Marza & Marza (24). Quart. Jour. Micros.
Sci., 78, 1935.

Bartelmez (2) found that the ovum had a very definite position of formation within the follicle and within the ovary. Two axes may be observed in the follicle, especially after pediculation; one through the nucleus and perpendicular to the surface of the ovary, and the other through the long axis of the follicle, since it is not spherical. On the perpendicular axis the nucleus and the germinal disc, with the latebra, are found from the ovary surface outward. The long axis lies on the anterior-posterior axis of the bird. The stigmata follows these axes and a bilateral symmetry is found along it.

Outside the follicle cells the adjacent stroma have a concentric arrangement forming the theca folliculi. Pearl & Boring (29) observed that this is separated into an inner

and an outer layer. Bartelmez (2) found a very vascular layer separating the two. A membrane propria has been observed that separates the follicular from the theca. Within the follicular epithelium is another fibrous membrane within which is found a zona radiata which is penetrated by numerous pores (Holl, 18) which are thought to contain protoplasmic filaments from the follicular cells which unite with the protoplasmic layer of the ovum underlying the zona radiata.

The vitelline membrane. The vitelline membrane as found on the egg yolk has been studied by Liebermann (21) and Lecallion (20). Liebermann (21) considered from chemical analysis that it was keratinous in composition. Lillie (22) states that the zona radiata is a primordium of the vitelline membrane. Lecallion (20) found that it consisted of three definite layers. The complete membrane is approximately 15 microns in thickness. The inner layer, toward the yolk, is the thinnest of the three, and is about three microns thick. The middle layer is two or three times as thick as the internal one. It is cellular in structure, the cells having an epithelial form. The nuclei of the cells are just recognizable, and the cytoplasm vacuolated. The external layer is fibrous. He also recognized small degenerate nuclei tangential to the surface of the ovum.

Atretic ovum. Certain ova throughout the growth period of the ovum, or at any age of the bird, begin to break down and be resorbed. These are called atretic follicles. The reason for this formation is unknown. Stieve (42) found

that large atretic follicles could be produced by taking
hens that had been laying daily and removing the feed for
24 hours.

Ovulation. While the exact stimulus necessary to cause
ovulation in the fowl has not yet been found several pertinent
facts have been noted relative to normal ovulation.

Warren & Scott (48) discovered that ovulation occurs on
the average 30 minutes after the laying of an egg, if another
egg is to be laid in the clutch. They found that it was not
a stimulus produced from the oviposition itself. This they
proved experimentally by the removal of the egg prematurely
from the uterus. Ovulation was found to occur at the regu-
lar time and not at the time of the removal of the egg.

Oviposition occurs with regularity in the domestic fowl
and depends upon the length of clutch. In eggs laid in a
long clutch, say ten eggs or more, oviposition will take
place at definite 24- to 25-hour intervals between eggs. At
the latter part of the cycle the interval will lengthen until
the last egg is laid in the afternoon. Eggs laid in short
cycles may show as much as 30 hours between eggs. Phillips
& Warren (30) found that the length of time between oviposi-
tion and ovulation was increased when the length of interval
between eggs was increased.

An inhibiting action seems to operate in the environment
on ovulation in the latter part of the day so that oviposition
may take place without ovulation. This means that a day is
skipped and a new ovulation takes place at such a time that
a new clutch is started in the morning hours.

Warren & Scott (48) found that under the influence of
continuous lighting the tendency to lay in clutches is re-
duced, ovulation and oviposition taking place at any time in
the 24 hours. That light or darkness affects the time of
ovulation was experimentally proven by the reversal of light
from the normal rhythm. By using artificial lights at night
in a room darkened in the day hens could be induced to lay
at night.

Phillips & Warren (30) were able to observe ovulation
by operating on hens under anaesthesia. They were unable to
bring about normal ovulation by increasing the pressure in
the ovum. Ovulation was found to occur even after the ovum
was removed from the ovary. They advanced the theory that
the normal rupture of the stigmata necessary for ovulation
was brought about by contraction of muscle fibers in the fol-
licle wall. They were, however, unable to cause ovulation
by electrical stimulation of the sides of the follicles.

EXPERIMENTAL METHODS AND PROCEDURES

Owing to the fact that the experimental work consists of three distinct phases, as outlined in the introduction, it would seem best for clarity and continuity, to give the experimental methods, results, and discussion of each phase independently.

Rate of Yolk Formation

Experimental procedure. New Hampshire and Barred Plymouth Rock hens were kept in laying batteries and fed the regular station mash for eight hours or more per day. In order to observe the rate of growth of the ova, the birds were given a gelatine capsule containing approximately 10 milligrams of Oil Red O mixed in one cubic centimeter of corn oil every 24 hours according to a method recently described by Denton (10). The Oil Red O dosage was administered throughout the experiment. The yolks showed a complete series of rings after hard-boiling and sectioning. They were cut in the approximate middle at right angles to the neck of the latebra, so that the neck would not interfere in the measurements. These measurements were made under the microscope, using the mechanical stage with a millimeter scale and vernier. Measurements were taken from the outer surface of the sectioned yolk to the inside of the first ring and between the inner sides of the following rings. A complete series of readings was made across the yolk and another at right angles, making four separate measurements of the

thickness of each layer. In making the calculations for the thickness of the daily increase in radius these four measurements were averaged.

Preliminary observations, before measurement of the rings was undertaken, were made to observe variation in the number of rings. All the eggs from twenty-two hens were opened for a period of more than three months in order to look for variation in number of rings. Layer thickness measurements were made for a period of over a month on eighteen hens.

Results: The number of layers found varied from six to ten. This difference in the number of rings is slightly more than might be expected from Riddle's (31) work, since he states that the normal rapid growth period is from five to eight days. Some birds, we noted, differed consistently in the number of rings, laying six- or seven-ringed yolks while the others produced yolks containing eight to ten rings. The yolk weight, however, remained approximately the same. Representative samples appear in Plate I, Figure 1, A, for the six-ringed and Figure 1, B, for the eight-ringed yolk. The difference in the thickness of the layers is very obvious, as demonstrated in Plate I.

The consistency of the thickness of ring, the constant number of rings, as a physiological characteristic of each bird is shown in Table IV, a representative sample of the measurements of the layers for a single bird.

Table IV. Measurements of daily increase in radius
of yolk of same bird

Yolk wt. gms.	Number of rings	Measurements of layer thickness from outer layer toward center (Average of four measurements in mm.)						
		1	2	3	4	5	6	7
15.6	10	0.55	1.35	1.43	1.68	1.85	1.40	1.45
17.9	7	1.23	1.20	1.60	2.08	2.03	4.93	1.53
16.0	7	0.88	1.60	1.85	2.48	1.83	1.83	1.65
17.1	7	0.98	1.18	1.43	1.83	2.13	3.08	2.35
16.1	7	.63	1.08	1.50	1.80	2.00	2.33	2.93
16.3	7	.58	1.93	1.35	2.03	2.68	2.25	2.27
16.0	8	.48	1.10	1.33	1.98	2.10	2.53	2.62
16.5	7	.48	1.28	1.45	1.70	2.68	2.43	2.08

The difference of the yolks of one hen from another in
the thickness of the layers is shown in Table V. The accumu-
lative radius increments for the same hens are plotted in
Chart II.

Table V. Measurements of daily increase in radius
of yolk of different birds

Av. yolk wt. gms.	Av. number of rings	Measurements of layer thickness from outer layer toward center (Average in mm.)						
		1	2	3	4	5	6	7
17.0	6.33	.89	1.31	1.53	1.93	2.29	2.64	1.95
16.4	7.75	.72	1.34	1.83	1.94	2.15	2.59	2.11
11.4	6.33	.41	0.90	1.23	1.71	2.19	2.60	2.62
16.5	9.00	.81	1.27	1.44	2.88	1.79	1.55	1.76

Discussion. In view of the conflicting reports by
Stieve (42) and Conrad & Warren (47) on the daily growth rate
during the final rapid-growth phase of the ovum we considered
it important to restudy the growth during this phase. As was
pointed out in the introduction, Stieve considered rate of
growth during the rapid-growth phase quite variable. His

CHART II

Cumulative Radius Increment
of Average Yolks for Four
Different Hens

Number of Layers
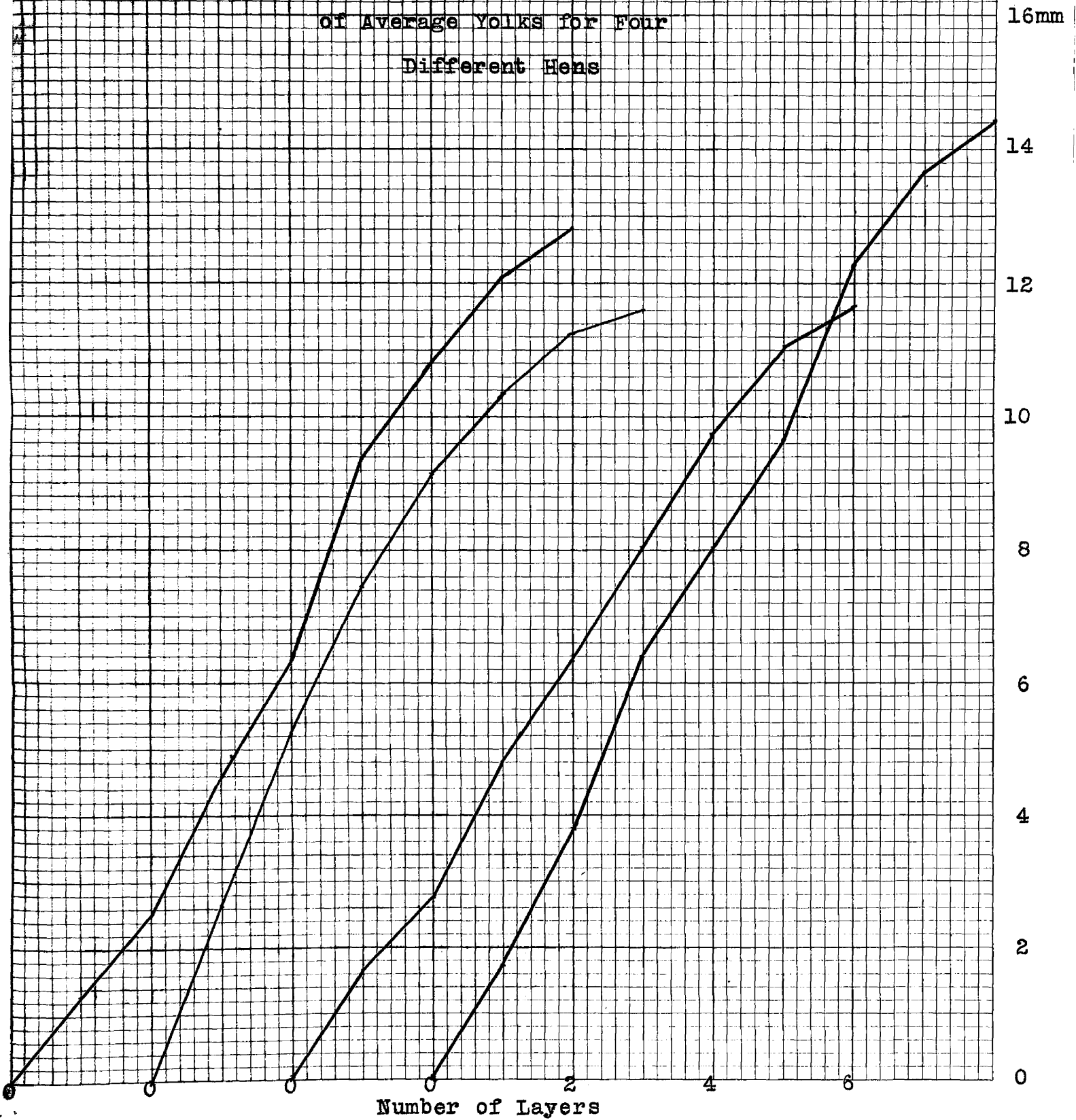
observations on the number of follicles in the rapid-growth phase in different hens were few. The number of follicles and their weight in the ovary would, moreover, give only an approximate picture of normal growth, since it would depend upon the future egg production of the bird, an unknown quantity. Warren & Conrad (47) state that differences in yolk size between the yolks of a single bird and yolks of different birds are probably not the result of more rapid growth. They conclude that larger yolks are produced by a longer period of growth. They did not attempt to measure the difference in length of time of the rapid-growth period.

As the experimental results point out, a difference in length of the rapid-growth phase was found for both the same hen and between one hen and another. This variation, while readily observed, is not extreme, being only from six to ten days. The length of the rapid-growth phase seems to be characteristic of the individual hen, although a variation of from six to ten days was found for different birds, the variation for a single hen was found to be not more than one or two days.

We attempted, by counting back from the day of ovulation, to find the number of follicles in the rapid-growth phase on different days in the same bird. We found a definite cycle of increase in number, which would correspond to a cycle of yolk formation. These yolk-formation cycles which precede a laying cycle may be due to increased activity of the pituitary gland of the hen. Laskowski (19) found an increase in

the blood of the serum-vitellin and phospho-lipoid fraction after the injection of the gonadotropic hormone from the pituitary. The remarkable stimulation of the follicle growth in the fowl by this hormone has been demonstrated by Bates, Lahr, & Riddle (4). Roepke & Hughes (37) found a similar increase of the serum-vitellin in the blood of active laying hens, while Roepke & Bushnell (36) identified the similarity between the serum-vitellin and the ova-vitellin. The largest number of follicles calculated from these experiments as present at one time was six. It should be possible, however, for a bird having a ten-day-period of rapid growth and laying daily to have ten follicles in the rapid-growth phase.

While the difference in rate of yolk formation per day is not extreme, more variation is noted than might be expected from the statements of Warren & Conrad (47). The differences in the cumulative increase in radius for the largest and smallest yolks of two different birds is plotted in Charts III and IV. They show the difference in the rate of yolk deposition in the ova.

In order to bring out this difference more clearly the average difference for daily increase in radius has been calculated for ten yolks heavier than 18 grams and for eight yolks of less than 15 grams. These calculations are given in Table VI and plotted as cumulative increments of the radius in Chart V. These computed data demonstrate clearly the fact that with a difference of 5.45 grams in average yolk weight the difference in the number of rings is very
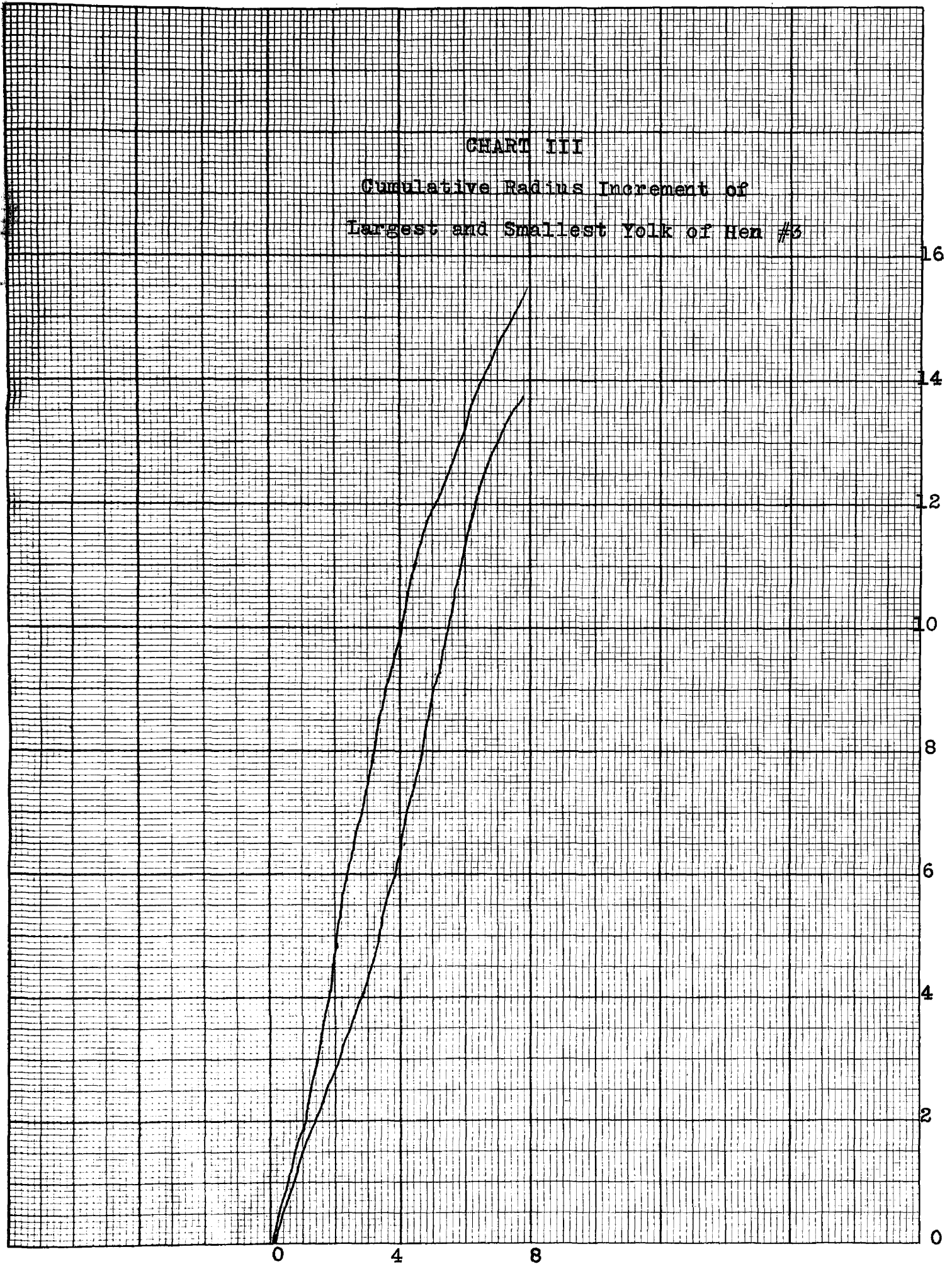
Chart III

CHART III

Cumulative Radius Increment of

Largest and Smallest Yolk of Hen #3

CHART III

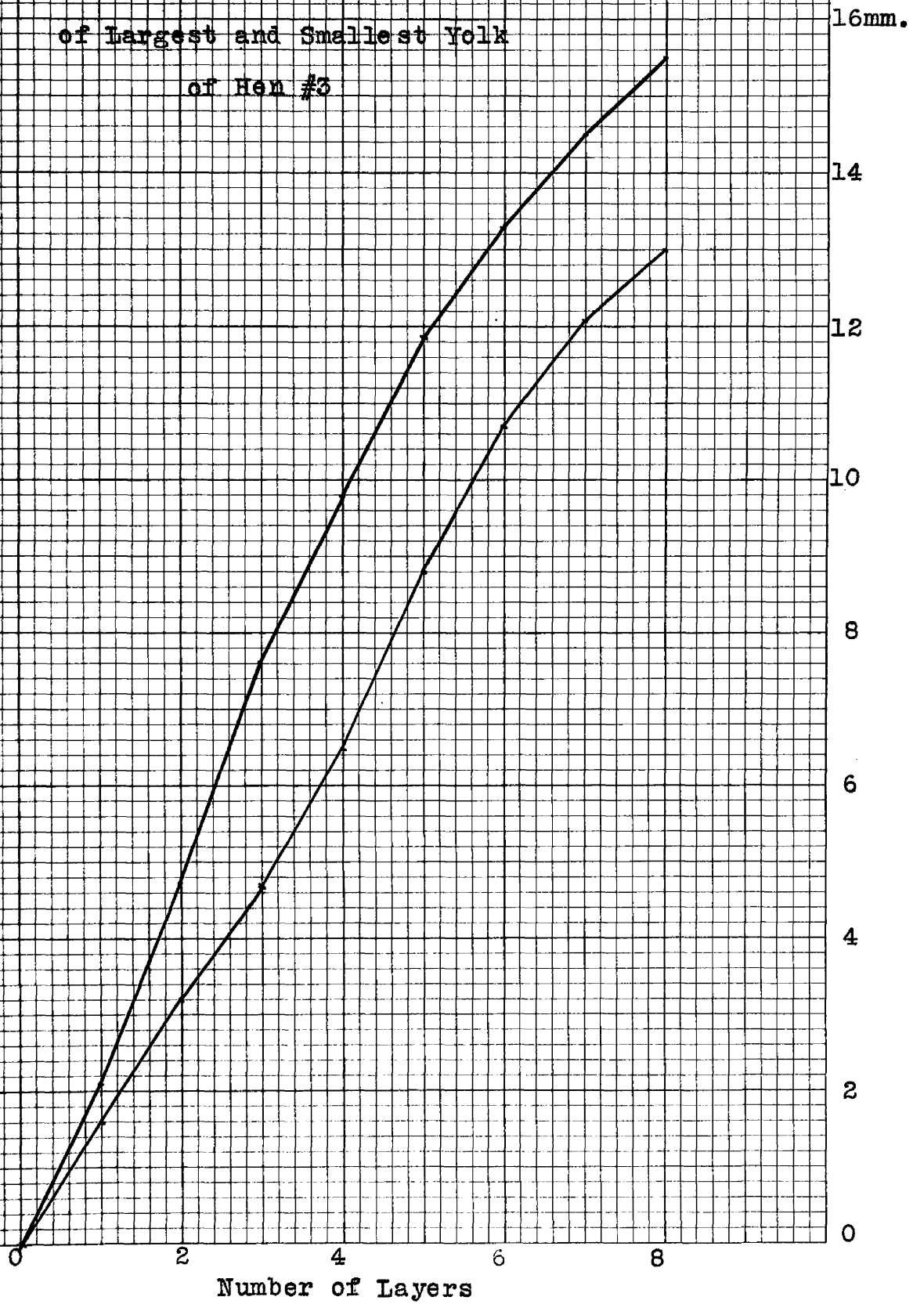Cumulative Radius Increment
of Largest and Smallest Yolk
of Hen #3

Number of Layers

CHART IV

Cumulative Radius Increment
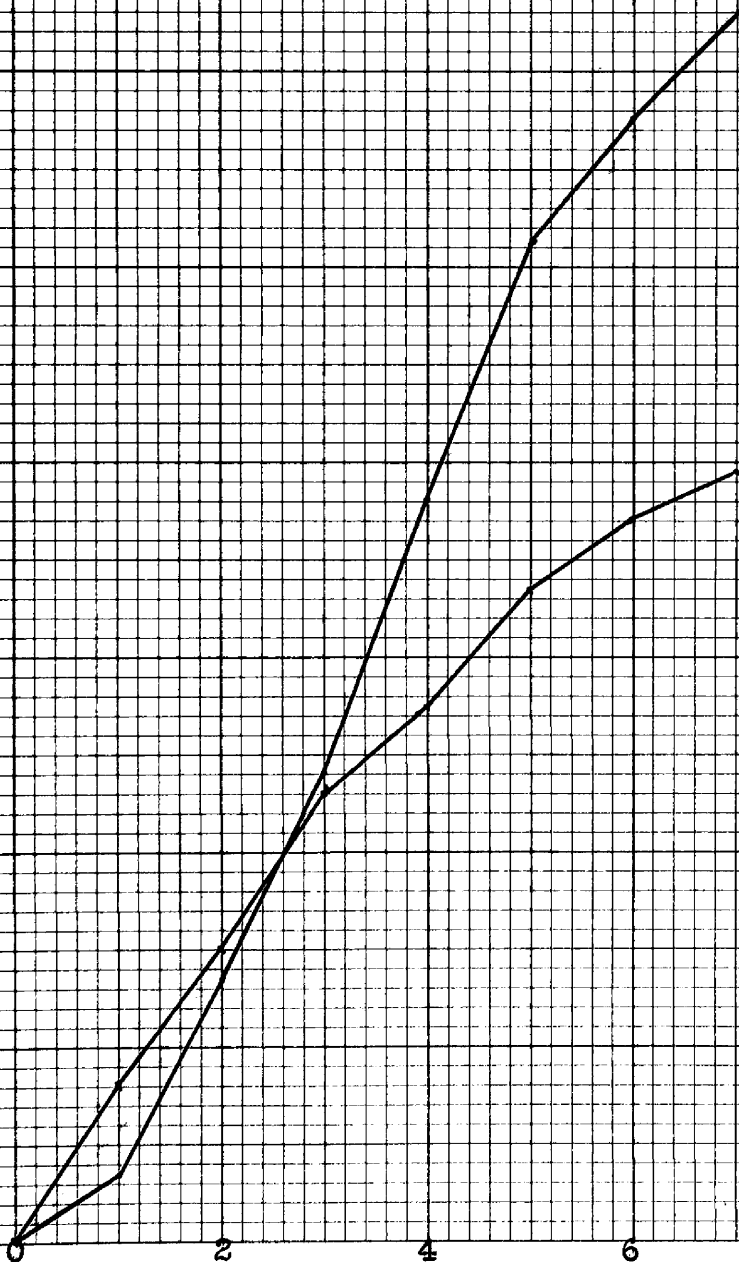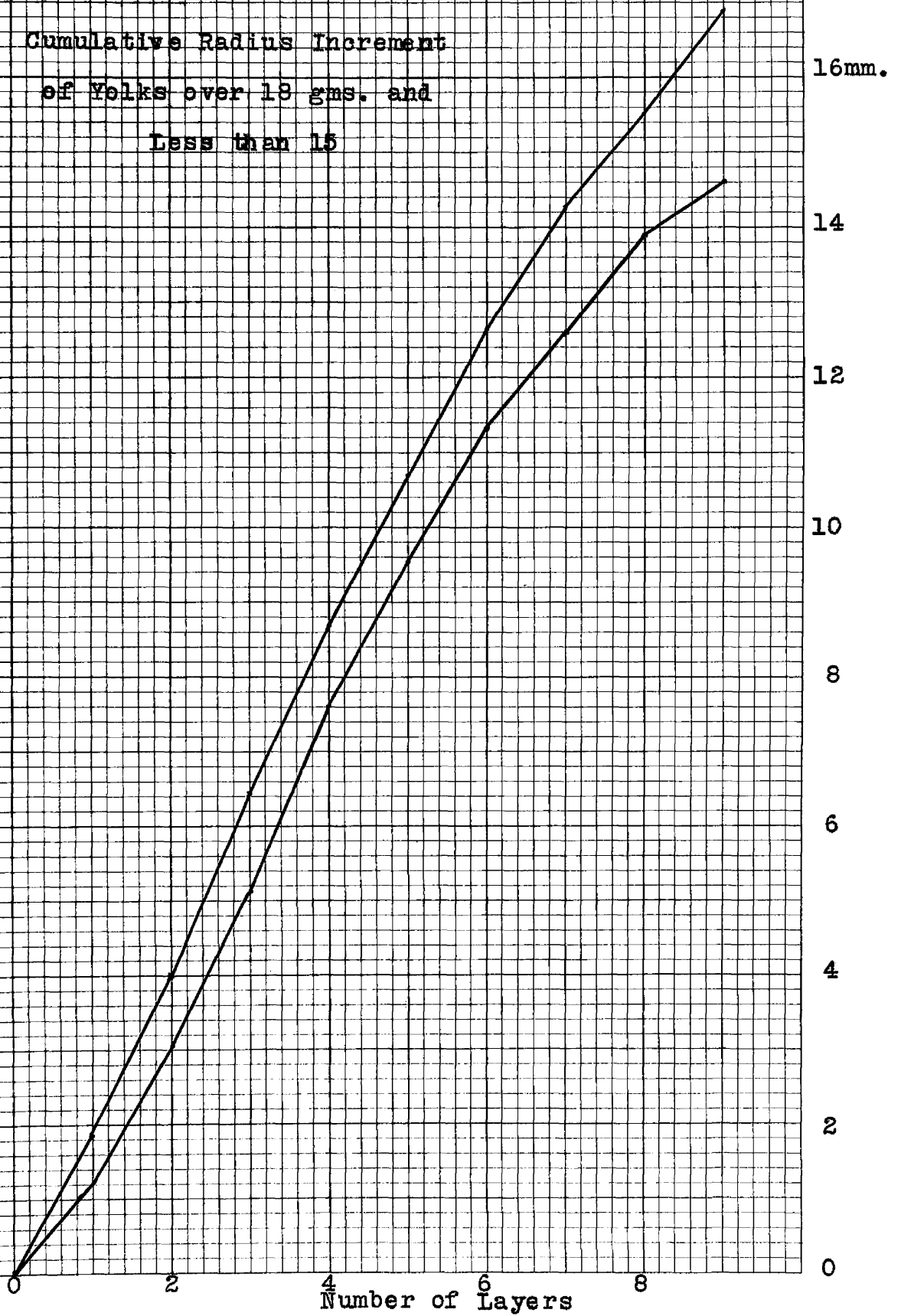of Largest and Smallest
Yolk of Hen #16

Number of Layers

CHART V

Cumulative Radius Increment
of Yolks over 18 gms. and
Less than 15

small, only 0.44 of a ring. The most important factor for yolk size is not length of rapid-growth phase but difference in daily rate of growth.

Table VI. Comparison of daily increase in radius of yolks averaging less than 15 grams and those averaging more than 18 grams

Av. yolk wt. gms.	Av. number of rings	Measurement of layer thickness from outer layer toward center (in mm.)							
		1	2	3	4	5	6	7	8
Under 15 grams									
13.14	7.06	0.68	1.27	1.33	1.74	1.92	2.46	2.12	1.86
Over 18 grams									
18.59	7.50	0.91	1.28	1.60	1.98	1.97	2.26	2.38	2.23

That the length of the rapid-growth phase is not a too sharply defined characteristic for the individual hen but may be subject to changes is shown in Chart VI. Here the number of layers in the yolk is found to decrease with an increase in the length of the clutch in which the yolk is formed. Since the yolk size is not reduced during times of heavy production an increased rate of yolk formation for the individual follicle and a tendency to reduce the number of follicles in the rapid-growth phase would follow.

While making the study of the rate of yolk formation observations were also made on stratification in the color during the rapid-growth phase. The statement of Gage & Gage (14) that stratification was found in yolks from hens being fed completely on feed containing Sudan III does not

CHART VI

Comparison of Number of Layers
in Yolk During Rapid Growth
Phase with Length of Clutch
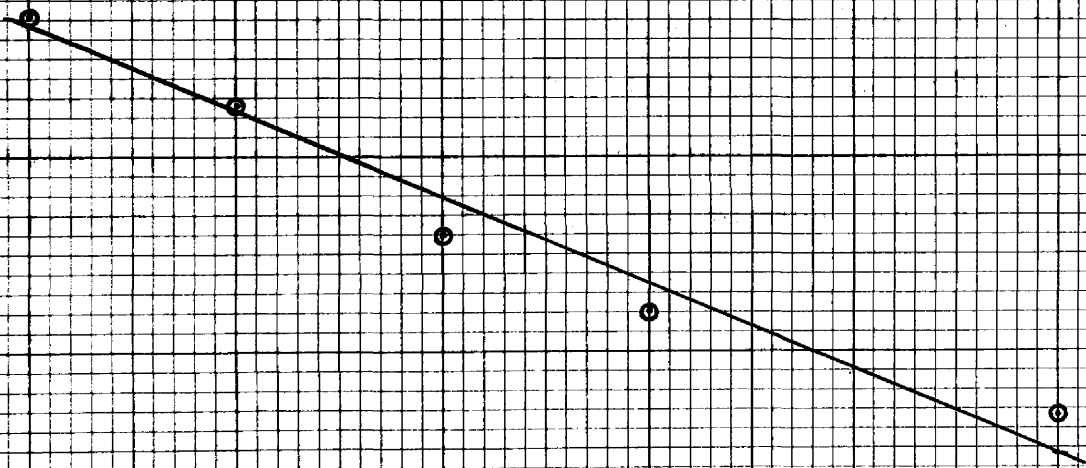
appear to be reconciliable with the conclusions of Conrad
& Warren (8) that stratification was caused by a short feed-
ing period or a short daily exposure to yolk-coloring pigments.

Hens were fed on a mash containing the dye Oil Red O.
The feed was before them at all times as they stood in the
battery. Representative yolks produced are shown in Figure 1,
C, and D. A very distinct stratification may be noted.
Other hens were placed on a ration containing 10 percent corn
gluten meal and 10 percent of high quality alfalfa leaf meal
known to carry yolk pigments. Yolks produced by these hens,
which were also kept in batteries, were also found to have
a slight stratification.

In some other hens stratification of yolk materials
could be observed even in colorless yolks, showing that some
basic difference in the yolk material might partially account
for the stratification of the color if it were present.

The difference in rate of absorption of fats from the
digestive tract from other material and the fact that as
found by Henderson & Wilcke (17), the hen is not able to
withdraw pigments from the body fat for inclusion in the egg,
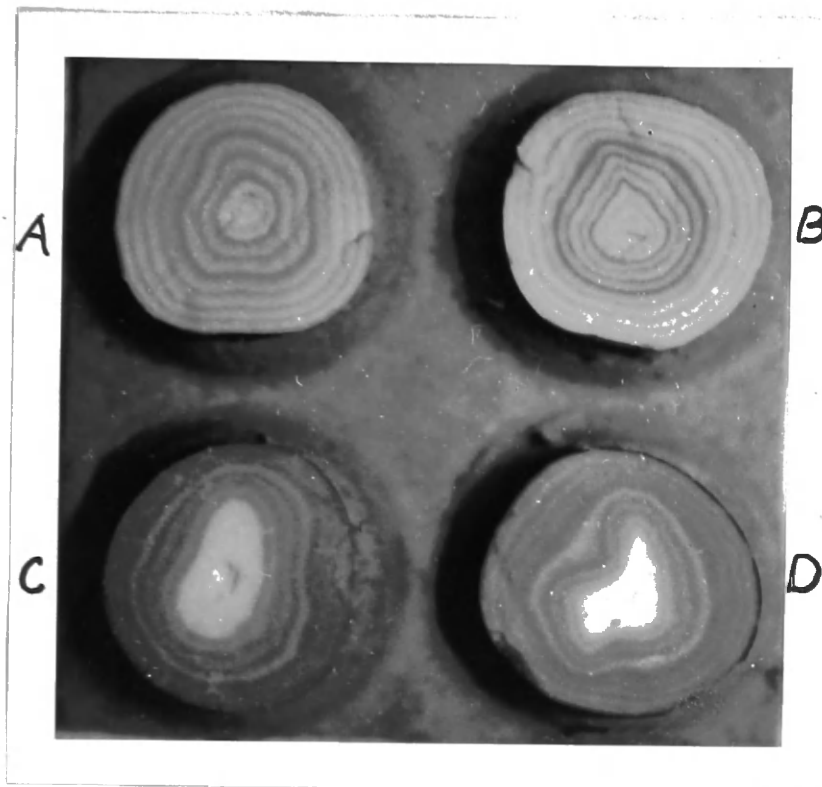may account for stratification of the pigment in the yolk.

PLATE I

Figure 1.   Yolks from Eggs Produced by
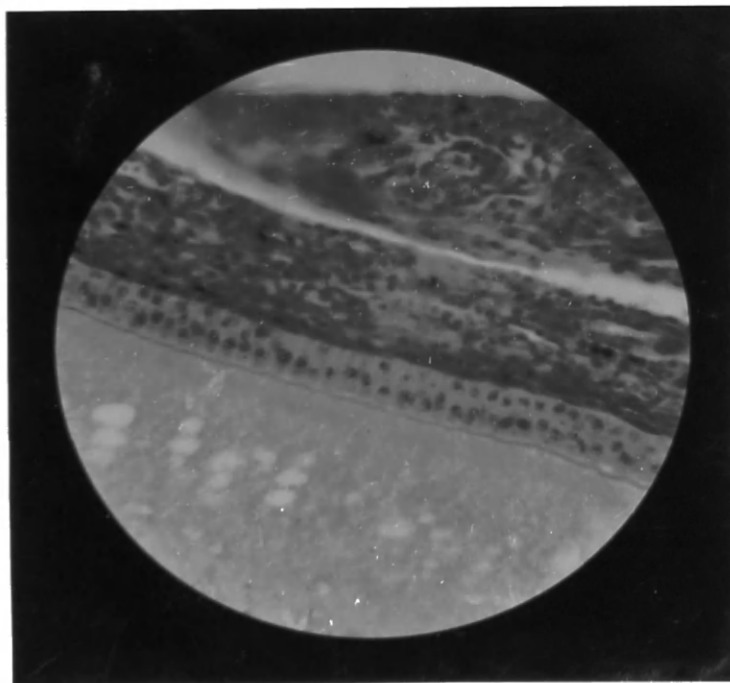Hens Fed Oil Red O

Figure 2.   Section of Large Follicle Wall.
Hematoxylin Stain

Origin of the Vitelline Membrane

Experimental methods. Microscopic sections were made
of the ovary, larger follicle walls, and the vitelline mem-
brane from yolks of eggs which have been laid, using the
customary paraffin-embedding method. These sections were
stained with alum hematoxylin and with Mallory's triple
stain. Some vitelline membranes were also obtained immedi-
ately after ovulation, in order to prevent any change caused
by the secretions of the oviduct that might occur in the mem-
brane. We also found it possible to obtain parts of the
membrane after artificial rupture of the follicle along the
stigmata.

Results and discussion. A typical section of the fol-
licular wall of a large ovum is shown in Plate I, Figure 2.
The pseudo-stratification of the follicular epithelium may
be observed as described by Marza and Marza (24). The thin
membrane lining the inner surface of the epithelium shows in
Figure 2. A membrana propria outside of the follicular epi-
thelium may be seen in some sections. Both of these membranes
were brought out more clearly by staining with Mallory's
triple stain, the membranes taking up the aniline blue of the
stain. In the theca interna and externa numerous connective
tissue fibers were also found to stain blue with Mallory's
triple stain. These fibers may be observed in Plate II,
Figure 1. Smooth muscle cells were not found in either the
theca interna or externa, as described by Phillips & Warren
(30). It is well appreciated how easily a few muscle cells
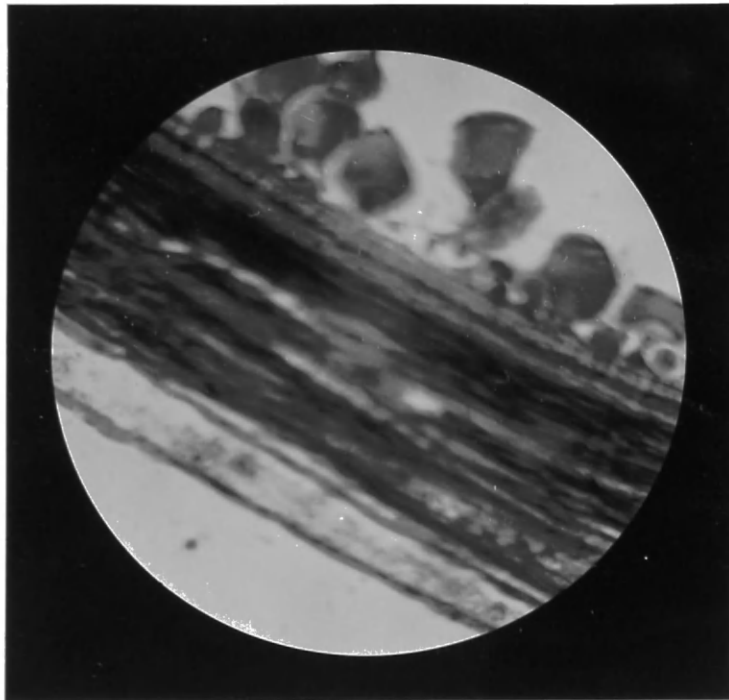
PLATE II

Figure 1. Section of Large Follicle Wall.
Mallory's Triple Stain
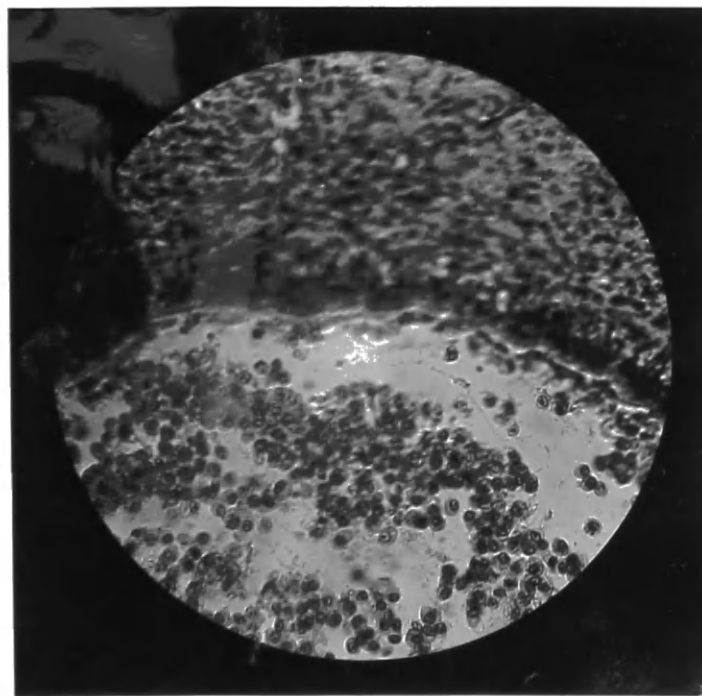
Figure 2. Section of Calix of Follicle
after Ovulation. Mallory's Triple Stain

might be difficult to find without special methods used for their identification in such a mass of collagenous fibers.

We found the sections made of the vitelline membrane of eggs did not contain cellular structure, contrary to the reports of Lecaillon (20), who claims to have found cellular structure between two fibrous layers. In order to further confirm the fact that perhaps the follicular epithelium with the two membranes on either side of it was not shed as the vitelline membrane, sections were made of the follicular wall after ovulation. The calix of the follicle was found to contain free in its cavity cells from the follicular epithelium and the membrane propria was still found on the follicle wall. This is shown in Plate II, Figure 2, thus leaving the inner collagenous fibrous layer as the only part that was removed with the ovum at ovulation.

We attempted to determine the character of the protein in the vitelline membrane by using the methods described by Almquist (1) in his study of the shell matrix. The vitelline membrane as found in the egg was not digested by pepsin in 1/10 N Hydrochloric acid solution, as would be characteristic of a collagenous membrane. Membranes from ova taken from the body cavity before being exposed to the secretions of the oviduct were found, however, to be digested by pepsin and soluble in 50 percent acetic acid, both characteristic of collagen.

We also made and measured sections of freshly ovulated yolk membranes. It was found that the membranes of the egg

yolks were approximately 100 microns in thickness, while the thickness of the freshly ovulated yolks was only 55 microns. Membranes from follicles that were removed by rupturing were even thinner, averaging only 42.5 microns.

From this work on the vitelline membrane, it was concluded that the membrane in the freshly ovulated yolk is of collagenous fibers. When the yolk is taken up by the oviduct the fibers swell, from the alkalinity of the secretions of the oviduct, a characteristic of the collagenous fibers, and the mucin secretions of the oviduct are also precipitated on the surface of the membrane due to its slight acidity. Romanoff (38) has pointed out that the ova have an acidity of pH 5.9.

These findings are of interest in regard to fertilization. The membrane is only a thin fragile collagenous layer at the time the sperm are thought to penetrate it. The later thickening, and toughening by the mucin fibers protect the yolk against rupture while in the oviduct.

Experimental Factors Inducing Ovulation

The fowl has been found to ovulate on an average within 30 minutes after oviposition. This tendency was studied by Phillips & Warren (30) and their findings confirmed by others. As was pointed out in the introduction the time of ovulation seems to be more fundamental than that of oviposition, and indirectly sets the time of oviposition. In this study we have assumed the time of oviposition to be approximately the same after a period of adjustment and the beginning of a new clutch. Previously confirmed experimental work seems to justify the assumption.

Experimental procedure. Twenty-four New Hampshire hens and Barred Plymouth Rock pullets were kept in a four-deck laying battery and fed the regular station laying mash. The batteries were located in a small isolated room of approximately 10 x 15 feet. The single window was completely covered over and the only light in the room was that produced by two 200 watt electric light bulbs in reflectors about five feet above the batteries. The lights were turned on and off manually. Feed and water were always available except when stated otherwise.

To determine the effect of feeding alone on ovulation we kept the lights on for the full 24 hours, feeding part of the birds for the 12-hour night period and the others for the 12-hour day period. Other birds having feed available for the entire 24 hours were retained for controls at the same time. In this way we hoped to eliminate any effect on ovulation

that might be brought about by light and darkness.

In a later experiment the birds were given a dark period each day to compare the relative strength of the effect of feeding with the effect of light and darkness. After the dark period part of the birds were fed for the first half of the light period and part for the second half of the light period.

Results. Hens exposed to light 24 hours daily with feed always available showed a tendency to lay at any time within the 24 hours. This result confirms the work of Warren & Scott (48) on the effect of continuous lighting.

When the hens were fed for half of the 24 hours they soon became accustomed to the daily rhythm. They rested and slept during the period when feed was not available. Some time was required for adjustment, but this was usually accomplished within a week or ten days.

A representative sample of the egg production in relation to the feeding period is given in Table VII.

Table VII. Egg production of hens under continuous lighting fed 12 hours daily

Night fed

D O N O O N N N O N N O N Q O O O O O O O O O O N N O O N N N

O N O O O F D O N N N N O N N N N O O D O O O O O O O O O O N D N

O D O O N N O D O O N O O O O O O O O O O O O O O O O O O O O O

Day fed

N D O O O O O O O O O O N D D D D D D O D D D D O O O O O

O D O D O D O D O D O D O O O O O O O O O O D O O O O O O O

O O D O D O N D O O N O D O D O O O D O D D O D O D O D O D O

D = egg laid during day. N = egg laid during night.

A tendency to lay during the period of feeding may be observed. The data on the effect of feeding under continuous lighting have been collected and summarized in Table VIII.

Table VIII. Summary of data on continuous lighting

Daily feeding period	Eggs laid at night	Eggs laid during day
24 hour ....:	51	36
8 A.M. - 8 P.M...:	23	52
8 P.M. - 8 A.M...:	73	21

In the experiment to compare the relative strength of light and darkness with feeding all the birds were given a dark period from 10 A.M. to 4 P.M. daily. Half of the birds were then fed from 4 P.M. to 11 P.M. and the other half from 11 P.M. to 10 A.M. Although this schedule left part of the birds with a short feeding period it was above the six hours necessary for egg production. The dark period during the day was used to break up any normal diurnal rhythms that might overshadow the effect of feeding.

For a short time at the beginning of the experiment, a few eggs were laid during the dark period. This, however, did not last long. Representative data on the effects of darkness and feeding on time of egg production are given in Table IX.

Table IX. Egg production of hens with dark period
and fed during part of light period

Fed in first part of light period																			
S	O	S	O	F	O	F	O	F	F	O	F	O	F	O	F	O	F	O	F
F	O	F	O	S	O	F	F	O	F	F	S	O	F	O	F	O	O	S	O
O	O	O	O	S	O	F	S	F	O	O	F	O	F	O	O	F	O	F	O
S	O	S	O	O	S	O	S	O	O	O	F	O	F	O	F	O	F	O	F

Fed in latter part of light period																			
F	F	F	O	F	F	O	S	S	F	F	S	F	F	S	O	O	O	O	O
F	F	O	O	O	S	O	O	S	S	F	O	S	F	O	F	O	F	O	O
F	F	S	F	F	O	S	S	F	O	S	F	F	O	S	F	O	S	F	O
O	O	O	O	O	O	S	O	O	S	F	F	O	S	F	F	O	S	F	F

All eggs produced in light period.
S = eggs produced when not fed.
F = eggs produced when on feed.

The data for this experiment have been collected and

summarized in Table X.

Table X. Summary of data with dark period and
fed during part of light period

Daily lighted period	Daily feeding period	Eggs laid at night	Eggs laid during day
4 P.M.-10 A.M.	4 P.M.-11 P.M.	72	33
4 P.M.-10 A.M.	11 P.M.-8 A.M.	59	41

Discussion. Warren & Scott (48) have shown that dark-

ness and light may control oviposition and ovulation. However,

the author thought that other factors or diurnal rhythms

might be a factor. Welsh (50) has pointed out that often

other diurnal rhythms than those due to light and darkness

may be found in animals, and that the effect of light is often to stimulate or inhibit activity.

Although a tendency for feeding to influence the time of ovulation was observed in the data with continuous lights, it was necessary to test the data statistically. This has been done by using the chi-square test.

If we assume that feeding does not have an influence upon the time of laying the same number of eggs should be produced in feeding and fasting periods. The chi-square test for twofold classification is applicable to these data. The observed frequencies are given in Table XI. Calculation of chi-square yields a value of $x^2 = 37.529$. This indicates that the probability of feeding not influencing time of ovulation is extremely small.

A similar analysis of the results with a darkened period is given in Table XII. (See page 34 for tables XI and XII.)

Calculation of chi-square gives a value of $x^2 = 15.711$. On the assumption that there is no effect on time of ovulation a value of $x^2 = 6.635$ would occur only once in 100 times. The value found, 15.711, indicates that the probability that there is no effect of time of feeding on time of ovulation is exceedingly remote.

It would appear from these results that darkness and light only influence ovulation through darkness preventing the bird eating. Although feeding has been found to be more important than light as a stimulus, one should be cautious against assuming that it was eating itself that was the

cause. Feeding and activity are so closely related that it
is possible that activity and not feeding may be the stimu-
lus for ovulation.

Table XI. Observed frequency of eggs laid during feeding
and non-feeding periods, by birds lighted 4 P.M.-10 A.M.

Time of feeding	Eggs laid 4 P.M.-11 P.M.	Eggs laid 11 P.M.-8 A.M.	Total
4 P.M.-11 P.M.	72	33	105
11 P.M.-8 A.M.	41	59	100
Total	113	92	205
Theoretical expected frequency			
4 P.M.-11 P.M.	57.9	47.1	105
11 P.M.-8 A.M.	55.1	44.9	100
Total	113	92	205

Table XII. Observed frequency of eggs laid at night and by
day by birds lighted 24 hours daily but fed at
night or by day respectively

Time of feeding	Eggs laid at night	Eggs laid by day	Total
Night	73	21	94
Day	23	52	75
Total	96	73	169
Expected frequency			
Night	53.4	40.6	94
Day	42.6	32.4	75
Total	96.0	73.0	169

SUMMARY

Experimental studies have been made in three different phases of the formation of the ovum of the domestic fowl.

The length of the final rapid-growth period was observed to vary between six and ten days. The length of the final-growth period seems to be characteristic for a hen at least while in a single phase of egg production. The length of the final growth phase was found to decrease with increase in clutch length.

The rate of yolk formation per day was found to be variable. While some variation in length of the final growth period was observed, the rate of yolk formation was found to be responsible for the greater part of the normal variation found in yolk weight.

The vitelline membrane was found to be formed from the collagenous membrane which lines the follicular epithelium. The collagenous membrane becomes swollen when in contact with the secretions of the oviduct. Mucin secreted by the oviduct is precipitated on the collagenous membrane which thickened and strengthened it.

Ovulation in the fowl was found to be stimulated by feeding. Hens exposed to continuous lighting were found to lay and ovulate within the daily period in which they were fed.

Hens exposed to a short daily dark period and fed only during part of the light period were found to lay and ovulate more in the feeding period. Activity and not eating may be

the stimulus for laying and ovulation. Darkness may inhibit
laying and ovulation because it reduces activity, and feeding
may stimulate laying and ovulation because it increases
activity. Darkness may only inhibit ovulation more effectively
than fasting because it more effectually reduces all activity,
some birds being as active in light when fasting as when
eating.

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