

## Morphological Observations on the Stigma of the Follicular Wall Concerning the Mechanism of Ovulation in Hens

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Received September 25, 1979  
(Figs. 1-15, Diagrams 1-3)

That ovulation is induced by the rupture of the stigma region of the follicular wall is well known as a fact. From the early times, many hypotheses have been postulated on the mechanism of the rupture of the stigma, including the muscular contraction theory of PHILLIPS and WARREN<sup>1)</sup>, the vascular degeneration one of NALVANDOV<sup>2)</sup>, and the supposition of an intra-follicular pressure by earlier researchers<sup>3)</sup>. However the true mechanism of the rupture is not fully clarified as yet. Recently, it was found out that a certain pituitary hormone is involved in the rupture of the stigma, and that LH plays the roll of a trigger for ovulation<sup>4-9)</sup>. Despite of these new knowledge, the question why rupture occurs limitedly in the region of the stigma remains completely unanswered. This suggests that the structure of the stigma is mechanically less strong than the remainder parts of the follicular wall. Until now most of the researchers who studied the structure of the follicular wall<sup>10-14)</sup> did not pay much attention to the characteristic structure of the stigma.

With these consideration in view, the present study was made to examine the structure of the follicular wall, particularly that of the stigma. Observations were made on the general histological structures, on the vascular supply, and on the arrangement of connective tissue fibers, as well as on the nerve innervation of the stigma region.

### MATERIALS AND METHODS

The materials used were the matured follicles of White Leghorn hens in laying. For light microscopy, they were fixed in a 10% formalin solution and prepared in usual paraffin sections. They were stained with hematoxylin and eosin, or azan staining.

For scanning electron microscopy, the surface of each layer comprising the follicular wall was exposed in the following manner for observation. The free surface of the stratum granulosum was exposed by removal of the perivitelline membrane. For this purpose, the follicles were fixed slightly in a 2.5% glutaraldehyde solution (pH 7.4) as a whole for 5 minutes. Then they were cut into small pieces of membrane, and the perivitelline membrane was stripped off gently from the follicular wall in a container filled with a physiological saline solution. The other layers of the follicular wall were exposed respectively by removal of the overlying masked layers in the same manner as above after fixing for 30 minutes. These treatments were done with needles as carefully

as possible under a dissection microscope. The prepared materials were refixed in the same fixative for 12 hours. After fixation, the materials were dehydrated through alcohol and dried by the critical point drying method, using CO<sub>2</sub>. Then they were coated with gold by means of an ion sputtering apparatus. These specimens were examined under a scanning electron microscope at an accelerating voltage of 15 KV.

For observation of the vascular architecture of the follicular wall, 30% gutta percha solution (used by dentists) of chloroform was used. It was injected into the descending aorta, after complete washing of the vessels with a physiological saline solution. Then the follicular walls were prepared in transparent preparation with glycerin.

For observation of the arrangement of the connective tissue fibers in the follicular wall, the follicular membranes were separated carefully with needles in thin stripped membranes under a dissection microscope. The separated membranes were stained by the BIELSCHOWSKY'S silver impregnation method for connective tissue.

For observation of the nerve innervation of the follicular wall, thin stripped preparations of the wall were prepared in the same way as mentioned above. They were stained by the BIELSCHOWSKY-GROS'S silver impregnation method.

### OBSERVATIONS

The follicular wall of matured follicles proved to be composed of the stratum granulosum, the theca interna, the theca externa, the connective tissue coat, and the superficial epithelium from inside to outside (Figs. 1 and 2). The stratum granulosum

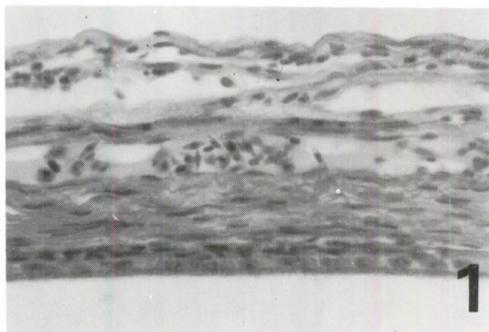


Fig. 1. Vertical view of the follicular wall of the non-stigma region. The wall is composed of the stratum granulosum, the theca interna, the theca externa, the connective tissue coat, and the superficial epithelium from inside to outside. The layer of the connective tissue coat is highly vascular. Hematoxylin and eosin staining. x420.

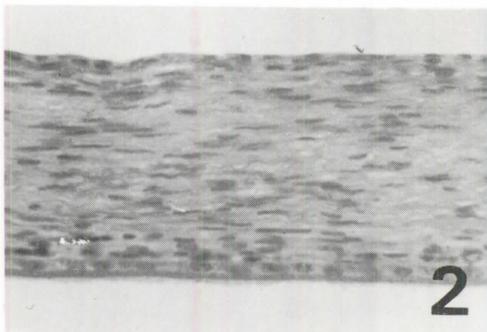


Fig. 2. Vertical view of the follicular wall of the stigma region. The theca externa comprises the greater part of the thickness of the wall. It intermingles compactly with the connective tissue coat. Hematoxylin and eosin staining, x 420.

lying innermost was a layer of granulosa cells surrounding the oocyte. The granulosa cells were cuboidal in shape and contained in their center a clear nucleus, histologically (Figs. 1 and 2). Just above the stratum granulosum, there was a well defined basement membrane which stained densely with azan staining. Under scanning electron microscopy (referred to as SEM), the granulosa cells looked somewhat spherical with distinct contours.

Most of them projected a long cytoplasmic process and provided numerous tall and slender microwilli on their free surface (Fig. 3). The basement membrane appeared as an amorphous thin membrane (Fig. 5). Since the stratum granulosum enclosed completely the oocyte, the granulosa cells showed no special structural difference in the region of the stigma.

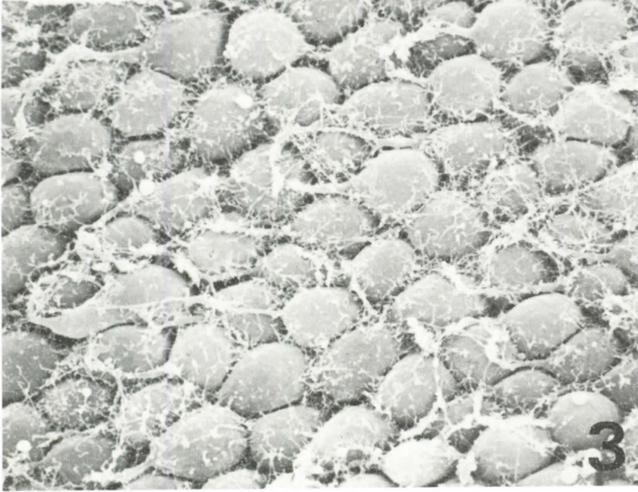


Fig. 3. Scanning electron micrograph of the stratum granulosum. Granulosa cells are cuboidal in shape. Each has a long cytoplasmic projection and numerous fine microvilli on its free surface. x 1,000.

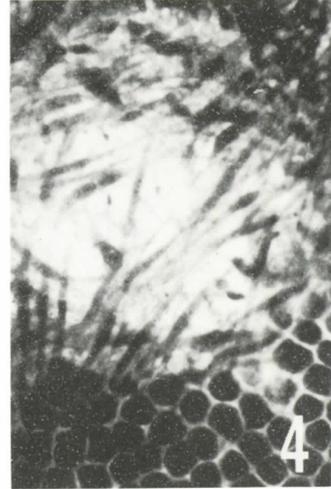


Fig. 4. Horizontal section of the follicular wall at the plane of the innermost stratum granulosum. The granulosa cells of the stratum granulosum and the smooth muscle-like cells are visible. Hematoxylin and eosin staining. x500.

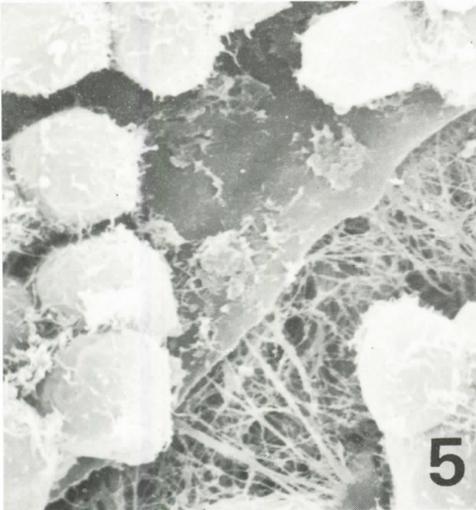


Fig. 5. Scanning electron micrograph of the basement membrane. The membrane is thin and amorphous. Just above it are granulosa cells and just beneath it is the theca interna. x 2,000.

The theca interna situated outside the stratum granulosum was a narrow zone of connective tissue containing capillaries and various cell elements in abundance. They were fibroblasts, fibroblast-like cells, and smooth muscle-like cells. Fig. 4 shows the appearance of smooth muscle-like cells in histological preparations. By SEM, the theca interna was grossly subdivided into two parts differing in structural appearance, an inner and an outer layer (Fig. 6). The inner layer was composed mainly of a network of

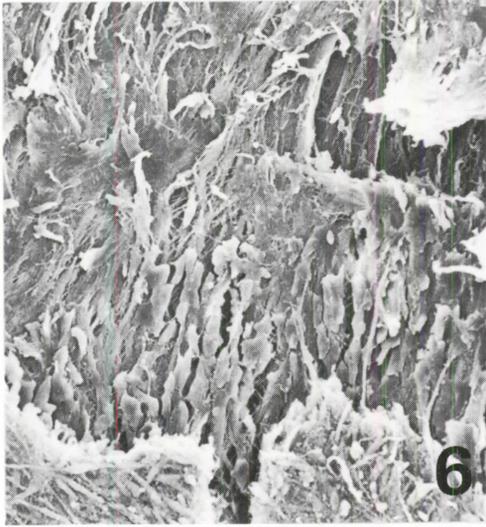


Fig. 6. Scanning electron micrograph of the theca interna. The lower portion of the figure is the inner layer of the theca interna. It is occupied by numerous smooth-muscle-like cells and fibroblast-like cells. The middle portion of the figure is the outer layer of the theca interna. It is composed of fibrous connective tissue. The upper portion of the figure is the theca externa. x 200.

capillaries and the arrangement of smooth muscle-like cells, which were long spindle-shaped and scattered at random amongst connective tissue fibers (Fig. 7). The networks of capillaries often contained a mass of closely packed erythrocytes. The outer layer was composed of a coarse layer of fibrous connective tissue containing polygonal fibroblast-like cells (Fig. 8). Connective tissue fibers ran wavy and often formed bundles of fibers. No structural difference was observed in the layer of the theca interna between the stigma and the remainder part.

The theca externa just above the theca interna comprised the greater part of the thickness of the follicular wall (Figs. 1 and 2). It was composed of a layer of dense fibrous connective tissue containing some fibroblasts. The fibroblasts in this layer appeared largely elongated and flattened, compressed amongst fibers (Figs. 1 and 2). Vascular networks in this layer appeared poor histologically, except for the small vessels crossing vertically into the layer. By SEM, the theca externa was formed by complicated networks of sheet-like bundles of collagenous fibers, which ran parallel to the surface of the follicle. These bundles of fibers showed vaguely because each fiber was cemented tightly with ground substances (Fig. 9). A few largely deformed fibroblasts were embedded among these bundles of fibers. The layer of the theca externa was formed uniformly in structure and thickness throughout the entire wall, except in the region of the stigma. At the stigma, it was so much thicker than in the other parts that it occupied almost all the thickness of the wall.

The connective tissue coat overlying the theca externa was a loose connective tissue rich in blood vessels, smooth muscles, and nerve fibers. It derived from the ovarian stroma and stretched through the stalk toward the distal pole of the follicle, forming several layers of broad connective tissue sheets (Figs. 1 and 2). Therefore, this layer was well developed in the proximal half of the follicle, but became thinner where it approached the stigma. At the margin of the stigma, it lost the accompanying blood vessels and abruptly entered in the stigma region. The connective tissue coat of the stigma fused compactly with its theca externa, so that it was hardly distinguishable (Fig. 2). The smooth muscle cells of the theca externa were arranged in sheet-like layers rather than in bundles and stretched along large vessels up to the equatorial region of the follicle. The

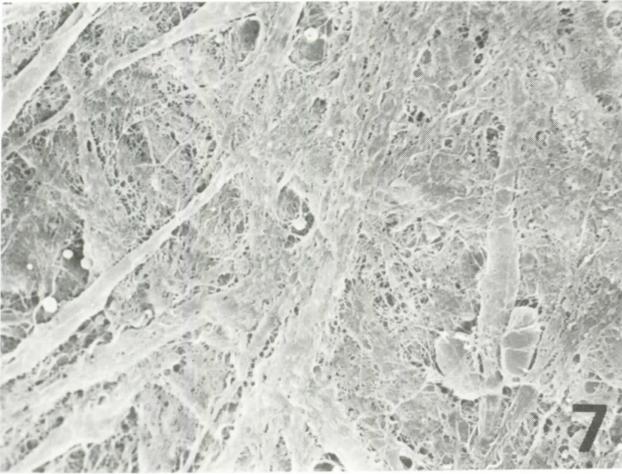


Fig. 7. Enlargement of the inner layer of the theca interna in Fig. 6. Long spindle-shaped smooth muscle-like cells are scattered amongst loosely arranged collagenous fibers. x1,000.

Fig. 8. Enlargement of the inner layer in Fig. 6. Abundant fibroblast-like cells are scattered. x1,000.



Fig. 9. Scanning electron micrograph of the theca externa. It is composed of compact sheet-like bundles of collagenous fibers. A broken nucleus of fibroblast is seen at the bottom of figure. x2,000.

blood vessels of this layer were mainly capacious large veins and formed complicated networks. In histological preparations, however, the stigma appeared to be devoid of blood vessels.

The superficial epithelium enclosing the whole outer surface of the follicle consisted of a single layer of flattened epithelial cells (Figs. 1 and 2). The appearance of the cells was varied under the intrafollicular pressure of the follicle. By SEM, the shape of the epithelial cells appeared to be squamous, and they were connected with each other by cytoplasmic projections (Fig. 10). This may enable the epithelium to stretch without breaking the cells.

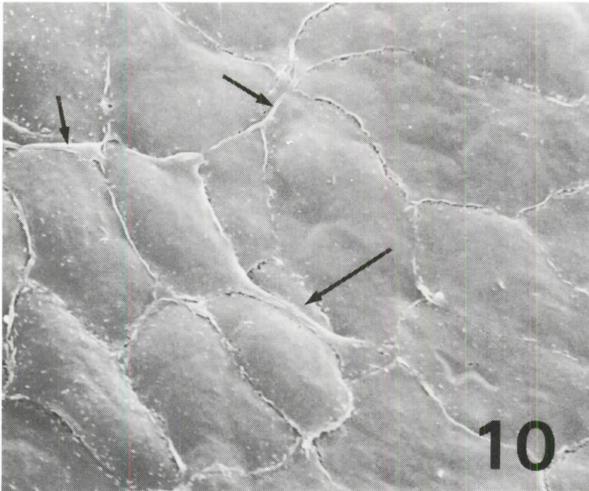


Fig. 10. Scanning electron micrograph of the superficial epithelium. Epithelial cells are flat and polygonal in shape. They are connected one another with cytoplasmic processes (arrows).  $\times 1,000$ .

By vascular injection of gutta percha, the vascular architecture of the follicle could be seen three-dimensionally. The follicular arteries which originated in the ovary became ramified into arterioles after they had gone through the stalk. These arterioles penetrated the theca externa and formed an arterial capillary network just beneath the stratum granulosum (Fig. 12). These capillary beds were collected into sinusoidal venules, and they penetrated vertically the theca externa (referred to as penetrating veins). After passing the layer of the theca externa, they came out in the layer of the connective tissue coat and drained into larger veins which formed a complicated venous network. Fig. 11 shows the distribution of the passageways through which the penetrating veins traversed the theca externa. As is shown in the figure, the passageways of the veins were represented as a leer hole. This figure was obtained by observation of the theca externa which remained after stripping of both the theca interna and the connective tissue coat from the follicular wall. The holes were distributed in the entire surface of the theca externa, but they were absent in the region corresponding to the area of the stigma (Fig. 11). Fig. 12 shows the vascular architecture around the stigma. As is shown in the figure, the stigma region was surrounded by a relatively large vein (referred to as circulating vein), which ramified in small branches downward with somewhat regular intervals. Those branches were not returning veins from the tissue of the stigma, but they were the penetrating veins that have been mentioned above. At the stigma, the penetrating veins stretched out upwards obliquely from the capillary beds of the theca interna, so as to escape the stigma. Therefore, the area of the stigma was highly avascular.

By the BIELSCHOWSKY'S silver impregnation method, the arrangement and its running

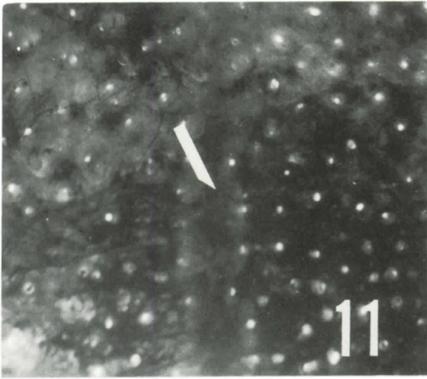


Fig. 11. Inner surface view of the stripped theca externa. It was made by removal of the theca interna and the connective tissue coat. Numerous holes indicate the passageways through which the penetrating veins traverse the theca externa. Notice that they are avoided in the region of the stigma (arrow). x6.



Fig. 12. Vascular architectures of the stigma. The stigma is surrounded by a circulating vein. It is received many penetrating veins from the theca interna with somewhat regular intervals. The capillary networks in the deep show the arterial capillary networks of the theca interna. Gutta percha injection preparation. x50.

mode of connective tissue fibers of the follicular wall was revealed clearly. In the layer of the connective tissue coat, arginophile fibers ran straightly or obliquely toward the opposite pole of the follicle from the stalk. In the theca externa, however, they were interlaced complicatedly, centering around the passageways of the penetrating veins mentioned above (Fig. 13). Whereas, those of the theca externa in the stigma were somewhat regularly interlaced lengthwise and crosswise. Particularly, longitudinal fibers running parallel to the long axis of the stigma were more dominant than transversal ones (Fig. 14).

The nerve innervation of the follicular wall was examined by the BIELSCHOWSKY-GROS's silver staining method. In general, the connective tissue coat of the follicular wall was richly innervated, especially in its half near the stalk. Those nerve bundles leading from the ovarian stroma through the stalk formed a complicated plexus with nerve cells and nerve fibers near the bottom of the follicle. The distal half of the follicle was poorly innervated and a few small bundles of nerve fibers ran across the stigma just beneath the superficial epithelium (Fig. 15).

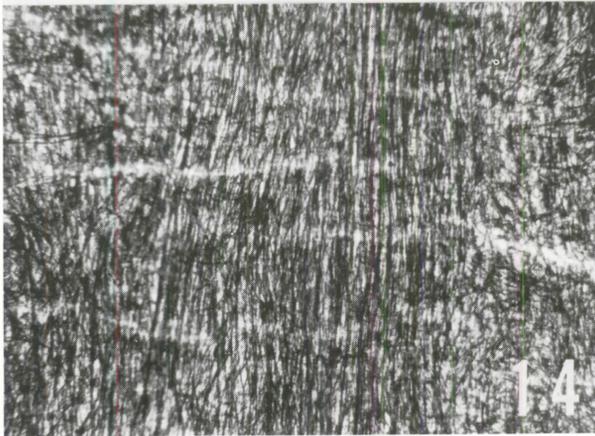
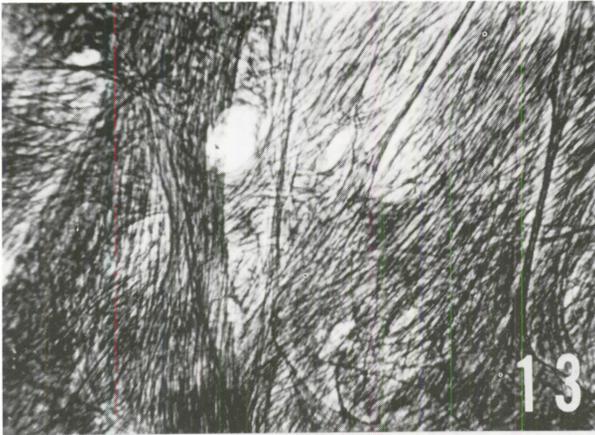


Fig. 15. Nerve fibers crossing the stigma at the just beneath the superficial epithelium. BIELSCHOWSKY-Gros's silver impregnation method. x420.

Fig. 13. The running of connective tissue fibers of the theca externa in the non-stigma region. The fibers are interlaced complicatedly, centering the holes showed in Fig. 11, BIELSCHOWSKY's silver impregnation method for connective tissue. x42.

Fig. 14. The running of connective tissue fibers of the stigma. The fibers run parallel to the long axis of the stigma. BIELSCHOWSKY's silver impregnation method. x42.

## DISCUSSION

This present study was carried out to observe the structure of the follicular wall, particularly the specialized structure of the stigma, where the rupture occurs by the time of ovulation. Since the general structures of the follicular wall have been examined microscopically and electron microscopically in detail by many investigators<sup>10-14</sup>), only characteristic points of the structure are discussed here. In accordance with previous authors, the follicular wall was composed of the stratum granulosum, the theca interna, the theca externa, the connective tissue coat, and the superficial epithelium from inside to outside. In our present study, however, a fine and three-dimensional structure of the follicular wall was revealed by scanning electron microscopy. Comparing the structure of the stigma and the non-stigma, no basic structural differences were observed. While some characteristic differences appeared in the arrangement of the connective tissue fibers, the

vascular architectures, and the nerve innervation.

Firstly, marked differences were observed in the layers of the connective tissue coat and the theca externa. Diagram 1 shows the general structure of the follicular wall.

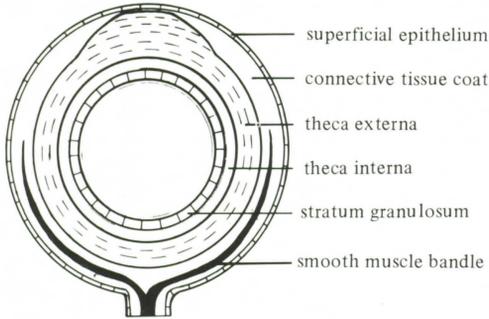


Diagram 1. General structure of the follicular wall.

Accordingly, the connective tissue coat of the stigma was very thin and compact, and intermingled with the layer of the theca externa. In contrast to this, the theca externa of the stigma was very thick and occupied the larger part of the follicular wall. The existence of the connective tissue coat, although thin, was evident because a few vessels and nerve fibers carried by the connective tissue coat ran across the stigma.

Secondary, the most striking structural difference between the stigma and non-stigma region consisted in the form of the vascular architecture. Many researchers<sup>15-18)</sup> have studied the blood supply in the follicular wall. NALVANDOV and JAMES<sup>15)</sup> first observed in detail the vascular architecture of the follicle by means of vascular injection preparations. Nishida *et al.*<sup>18)</sup> examined the microvascular architecture of the theca interna. It is generally accepted that the blood in the theca interna is supplied by a starfish-like network of arterial capillaries, and that its returning veins penetrate vertically the theca externa. Diagram 2 shows the outlines of the vascular architecture of the follicular wall obtained through this present study. As is shown in Diagram 2, the penetrating veins traverse vertically the theca externa in the non-stigma region. However,



Diagram 2. Outlines of the vascular architecture of the follicular wall.

As shown in Diagram 1, the layer of the connective tissue coat, which derived from the ovarian stroma through the stalk, became abruptly thin and went further on into the stigma. This was due to the disappearance of blood vessels, which formed complicated vascular networks in the layer of the connective tissue coat, at the edge of the stigma.

they are absent in the area of the stigma. In addition, the penetrating veins lying along the edge of the stigma penetrate obliquely the theca externa so as to avoid the stigma region. Accordingly, the region of the stigma is formed in an opposite-trigonal, avascular region, although only a few small vessels of the layer of the connective tissue coat cross it. This structure of the stigma may be compared to the opening of a vascular-basket. Owing to these specialized structures, the area of the stigma seems to be weaker mechanically than the non-

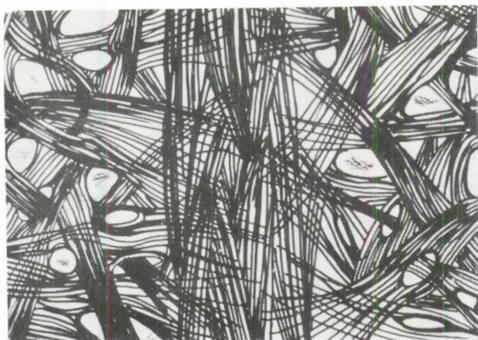


Diagram 3. Running mode of the connective tissue fibers of the theca externa.

stigma parts. This vascular architecture may serve to prevent hemorrhage at the time of rupture of the stigma.

Thirdly, the running mode of connective tissue fibers of the follicular wall was quite different in the stigma and the non-stigma. Diagram 3 shows the running mode of the connective tissue fibers of the theca externa. As is shown in Diagram 3, collagenous fibers of the theca externa form a complicated network in the non-stigma, whereas

they are interlaced rather longitudinally in the stigma along its long axis. These regular arrangement of fibers in the stigma seems to be attributed to absence of the penetrating veins mentioned above. These characteristic networks of fibers strongly suggest that the area of the stigma is much weaker mechanically than the non-stigma, and that the stigma is apt to split along its longitudinal axis. Actually, the rupture occurs in the longitudinal direction of the stigma at the natural ovulation.

The nerve innervation of the follicular wall has already been observed by many researchers<sup>19-21)</sup>. GILBERT<sup>19)</sup> has examined it with section preparations, and ORIBE and FUJII<sup>21)</sup> examined with wholemount thin ones. Their observations, however, were limited to the stalk and its neighborhood. In this present study, the stigma was less innervated than the non-stigma. Only a few small nerve fibers were present in the stigma.

From the above morphological observations, it was found out that in the whole wall of the follicle the region of the stigma was mechanically the weakest in constriction. As a result, it was concluded that rupture occurs in this limited area of the stigma of the follicular wall at the time of ovulation.

### SUMMARY

The structure of the follicular wall of hen, particularly the one of the stigma was examined in connection with ovulation. The morphological subjects of this study included the general and fine structure, vascular architecture, nerve innervation, and arrangement of connective tissue fibers.

The follicular wall enclosing the oocyte was principally composed of the stratum granulosum, the theca interna, the theca externa, the connective tissue coat, and the superficial epithelium from inside to outside. The structural difference between the stigma and non-stigma consisted in the theca externa and the connective tissue coat. In addition, the most striking differences were observed in the vascular architecture, the running mode of connective tissue fibers, and the innervation of nerve fibers.

The theca externa of the stigma showed a thicker construction than that of the non-stigma. In contrast, the layer of the connective tissue coat of the stigma was thinner and compacter than that of the non-stigma.

The blood supply of the stigma was very poor, although some small vessels were present. Particularly, the theca externa of the stigma was entirely devoid of penetrating veins, which arise from the capillary beds of the theca interna and penetrate vertically into the theca externa.

The arrangement of the connective tissue fibers was simpler and more regular in the stigma than in the non-stigma. The collagenous fibers of the stigma tended to run parallel to the long axis of the stigma.

The nerve innervation of the stigma was poorer than that of the non-stigma. These characterized structures of the stigma wall suggested that it is formed with less mechanical strength than that of the non-stigma, so that the rupture of the follicle occurs limitedly in the region of the stigma at ovulation.

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## 鶏の卵胞スチグマ部の形態的観察、 とくに排卵機構との関連において

藤井俊策・吉村幸則

鶏の排卵は卵胞スチグマ部の破裂によって行われる。卵胞壁がスチグマ部に限局して破れるのは、この部が何らかの構造的特異性をそなえているためと考えられる。本研究は卵胞壁、とくにスチグマ壁の構造を、一般的構造、血管構築の様態、結合組織線維の走行、神経分布などの諸点から調べた。

その結果、卵胞壁は基本的には内側から外側に、顆粒層、卵胞膜内層、卵胞膜外層、疎性結合組織層、漿膜層から構成されていた。

これらの諸層のうち、スチグマ部においては卵胞膜外層が最も厚く、疎性結合組織層が最も薄く構築されていた。他の層はスチグマ部と非スチグマ部の間には構造的差異は認められなかった。

卵胞壁の血管分布は密性であった。卵胞動脈は卵胞柄を通して卵胞に進入し、卵胞膜外層を横切って卵膜内層に達し、ここに密性の毛細血管床を形成していた。この血管床からの静脈は、貫通静脈となって卵胞膜外層を垂直方向に貫通して疎性結合組織層に進み、この層に吻合に富んだ静脈血管網を形成していた。しかし、スチグマ部の疎性結合組織層にはほとんど血管を欠いていた。また、卵胞膜外層には貫通静脈も欠いており、卵胞膜内層からの貫通静脈はスチグマ部を避けるように斜め方向に進み、スチグマ部を輪状に囲む静脈に吻合していた。したがって、スチグマ部は卵胞壁のうちで特徴的に血管に乏しい部分であった。

卵胞壁の主部を占める卵胞膜外層の結合組織線維の配列、走行は、非スチグマ部では著しく複雑で網眼状に交雑していた。これは貫通静脈の横断によって、線維配列が乱れたためであった。しかし、スチグマ部では貫通静脈を欠いているために、結合組織線維の走向は縦と横に比較的規則正しく配列し、とくにスチグマの長軸に走る線維が優勢であった。

スチグマには神経分布はほとんど認められなかった。

以上のような所見は、スチグマ部が卵胞壁のうちで物理的に最も弱い構造をしていることを示唆した。排卵時にスチグマ部が限局して破れるのは、このような理由によるものと考えられる。