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Studies on ovaries of mosquitoes using light and scanning microscopy

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Abstract
Mosquitoes are threatening the human population as these acts as the vectors for various diseases such as malaria, dengue, yellow, zika and west nile fever as well as lymphatic filariasis. The knowledge on the biology of mosquitoes is essential for a better control of both the insects and diseases they transmit. The present study aims to increase the knowledge on the biology of vector mosquitoes. It focuses on the ovaries of mosquitoes using light and scanning microscopy. The mosquitoes used in this study were composed of six different strains, namely: *Culex pipiens* Complex, *Aedes aegypti*, *Aedes vexans*, *Ochlerotatus cantans*, *Ochlerotatus rusticus* and *Anopheles maculipennis*. These were collected from different breeding sites in the Philippines and Germany. In light microscopy, abdomens of the female mosquitoes (from 2nd to 6th segments) which contained the paired ovary were detached from the rest of the body and were subjected to the standard procedure for fixing. In scanning microscopy, whole individuals of female mosquitoes were fixed. Results in light and scanning microscopy showed that two clusters of ovarioles are located in the ovary. These clusters of ovarioles are situated centrally and are surrounded with spongy fat body which encircles the abdomen beneath the cuticle. A presumptive follicle is joined to each maturing follicle in the region of nurse cells. The oocyte in which the nucleus is surrounded by a refractile lipid droplets is observed within the ovariole in a clear, non- staining lipid inclusion in its cytoplasm. Nurse cell is also present besides the oocyte. At the periphery of the oocyte, a light region is prominent which is just within the follicular epithelium. This region is the zone of yolk protein uptake. A meal of blood or controlled diet leads to a series of changes in cell structures in the reproductive organ of female mosquitoes that quickly results to the formation of matured eggs. One of the prominent changes observed in the ovary of mosquitoes having blood meal is the presence of various un-oriented microvilli in the area of the oocyte which is adjacent to the epithelial follicles.

Keywords: *Aedes aegypti*, *Aedes vexans*, *Ochlerotatus cantans*, *Ochlerotatus rusticus* and *Anopheles maculipennis*, light microscopy, scanning microscopy

Introduction
Majority of insects including mosquitoes reproduce sexually. Within the gonads of the ovaries, gametes differentiate and mature through a sequence of events that involves cells in complex structural and physiological changes (Smith, 1968) [14]. Female mosquitoes have two symmetrically arranged ovaries which are located parallel to the alimentary canal in the abdomen. These ovaries are located between the fourth and sixth segments of the abdomen (Roth & Porter, 1964) [11].

Mosquito ovaries are classified as meroistic type because they contain nurse cells as well as oocytes. Furthermore, they are categorized as polytrophic because groups of nurse cells are enclosed with an oocyte in each ovarian follicle. In contrast to other insects, like for instance mayflies (Ephemeroptera) and cockroaches (Blattodea), no nurse cells are present within the oocytes. This ovary is classified as telotrophic meroistic type. In these insects, the follicle Xcells are equipped to carry out secretion and protein synthesis (Smith, 1968) [14].

A central chamber or calyx is present in each paired ovary. The calyx is lined by an epithelium which contains muscle filaments that are also present in the lateral oviduct. Two tracheae enter each ovary and branch into the tracheoles within the ovarioles. Each paired ovary consists of variable numbers of tubular epithelial ovarioles where the oocytes are placed in a linear sequence according to their stage of growth (Smith, 1968) [14].

The number of ovarioles ranges from 50 to 500 depending on the species, physiological stages as well as the size of the individual female (Clements, 1992) [7]. Each of the ovariole is enclosed by a sheath which consists of cells forming a thin squamous mesothelium. Proximally, the ovarian sheath forms the long suspensory ligament which inserts into the 4th

abdominal tergite. The ovariole contains a germarium as well as a maturing and presumptive follicle. Differentiated cells of germarium through which the mitotic divisions of germ cells takes place resulting in the formation of the oocytes are observed in the anterior part of each ovariole.

In mosquitoes, these are accompanied by other cells which later perform the task of nursing or supplying nutrients to the developing gametes. In other insects, however, special nurse cells are absent and instead the nutritional responsiblity lies upon the epithelium of the oocyte follicles (Smith, 1968) [14]. The vitellarium which is composed of increasingly large and advanced oocytes, each is located within a follicular sac lies in the posterior portion of the ovariole. When the oocyte is fully mature, it passes into the oviduct. Each of the follicle contains seven nurse cells and one oocyte within the follicular epithelium and the youngest follicle cells "oogonia" lie in the distal position (Roth & Porter, 1964) [11]. The germarium, nurse cells, follicles and follicular stalks which compose each ovariole are invested by a basal lamina, which is sometimes called tunica propria (Bertram & Bird, 1961; Anderson & Spielman, 1971) [2, 1].

The common oviduct as well as the posterior parts of the lateral oviducts and most of the accessory glands are ectodermal in origin, whereas the real reproductive organ, the ovaries, are of mesodermal origin. The ovaries develop from a special section of splanchnic mesoderm, which is called the genital ridge. This ridge bears the germ line cells, which are sometimes segmentally separated into smaller groups.

Female mosquitoes have various sources of food during their life period. For the regular metabolism, they are feeding on flowers and fruits as a source of energy (e.g. for flying). However, for the development of eggs, they usually need a blood meal as a source of protein.

The physiological and structural changes that occur after having a blood meal are still under intensive investigations. Ultrastructure is the nanostructure of a biological specimen, such as a cell, tissue, or organ, at scales smaller than can be viewed with light microscopy. It is viewed with ultra-microscopy or electron microscopy. In this study, the ultrastructure of mosquito ovaries with and without blood meal has been studied by using light and scanning microscopy.

Materials and Methods

Mosquito strains

The mosquitoes used in this study were composed of six different strains, namely: *Culex pipiens* Complex, *Aedes aegypti*, *Aedes vexans*, *Ochlerotatus cantans*, *Ochlerotatus rusticus* and *Anopheles maculipennis*. These were collected from different breeding sites in various localities (Table1).

Table 1: Breeding sites and localities of mosquito strains mosquito strain type of breeding site locality

Mosquito Strain	Type of Breeding Site	Locality
Cx. pipiens	stagnant canal	Wittenweier, Germany
Ae. aegypti	old tire	Cebu City, Philippines
Ae. vexans	flood plains	Speyer, Germany
Oc. cantans	swampy woodlands	Hassloch, Germany
Oc. rusticus	swampy woodlands	Hassloch, Germany
An. maculipennis s.l.	semi-permanent water body	Bobenheim-Roxheim, Germany

Collection of mosquito samples

Mosquito samples were collected during larval stage. The collection was done by scooping the mosquito larvae with a fine mesh net. The larvae were placed in a small jar bottles containing water and covered with screens or fine nets.

Light microscopy

The abdomens of the female mosquitoes (from 2nd to 6th segments) which contained the paired ovary were detached from the rest of the body. Samples were fixed in Bouin's fluid for a period of 24 h and were dehydrated through a graded series of ethanol (70%, 80%, 90%, 96% & 100%). Before the samples were embedded two times in paraplast liquid, they were kept three times in a mixture of methylbenzoate (Intermedium alcohol/paraplast) and mounted by using a paraplast. By means of a microtome, sections of 6 µm thick were cut. The sections were stained by three different kinds of stains, namely; haematoxylin eosin and two different special stains from Goldner and Azan. After staining, the sections were mounted permanently by using depex as a mounting medium and covered permanently with glass slips and photographed under light microscope.

Scanning microscopy

The whole individuals of male and female mosquitoes were fixed for scanning microscopy. Fixing was done by using a primary cold fixative of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for a period of 2 h. This was followed by a buffer rinse of 0.1 M cacodylate three times for a period of 30 min. Secondary fixation was done by using 1% osmium tetroxide for two h. Samples were rinsed again 3 times with 0.1 M cacodylate buffer for a period of 30 min. Following the appropriate fixation method, the mosquito ovaries were dehydrated for 30 min. through a graded series of acetone (50%, 70%, 80%, 90% & 100%). Dehydrated specimens were transferred from absolute acetone to a critical point drying apparatus. The dehydrated specimens were placed in the specimen drying chamber of the apparatus. Enough absolute acetone was poured into the chamber to prevent air drying prior to critical point drying. The cover of the specimen chamber is secured and liquid carbon dioxide is added to the chamber from the tank. Acetone and carbon dioxide were mixed freely and the process was repeated eight times until carbon dioxide was only remained in the samples. The critical temperature of carbon dioxide is 31°C and 73.8 bar until the samples were totally dried.

Mosquito specimens dried by the critical method were mounted on stubs. Specimen tubs were coated with a thin layer of silver conducting paint and were allowed to dry in the petri dishes for overnight. The samples were then spotted with gold spotted SCD005 Baltec and were photographed using SEM 505 Philips.

Results

Light Microscopy

Two clusters of ovarioles are located in the ovary. These clusters of ovarioles are situated centrally and are surrounded with spongy fat body which encircles the bdomen beneath the cuticle (Fig. 1A). A presumptive follicle is joined to each maturing follicle in the region of nurse cells (Fig. 1B). Within the ovariole, a clear, non- staining lipid inclusion in its cytoplasm is observed. This is the oocyte in which the nucleus is surrounded by a refractile lipid droplets (Fig. 2A). Nurse cell is also present besides the oocyte. At the periphery of the oocyte, a light region is prominent which is just within the follicular epithelium. This region is the zone of yolk protein uptake (Fig. 2B).

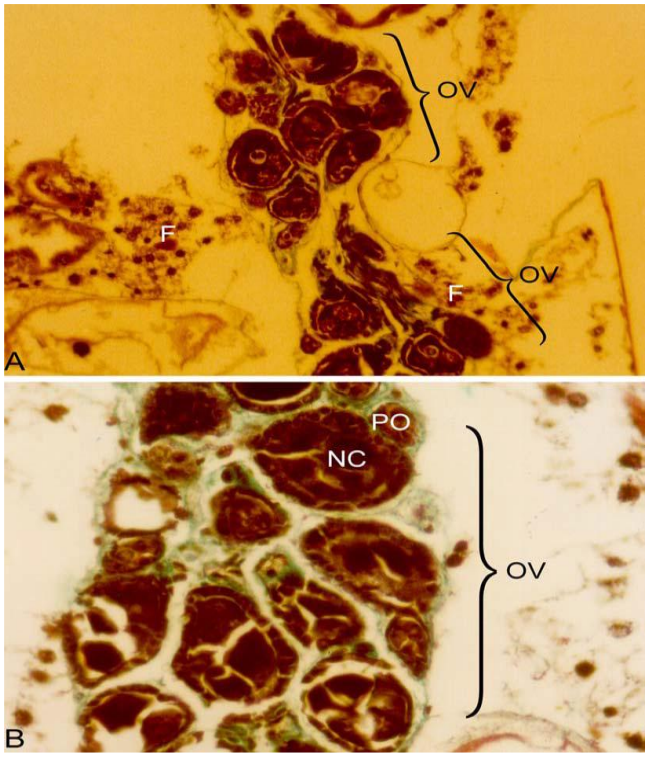

Fig 1: A Light micrograph of the ovary of *Aedes aegypti* showing two clusters of ovarioles (OV) with spongy fat body (F) which surrounds the entire clusters of ovarioles of *Aedes aegypti.* X2 500 B. An ovariole (OV) showing the presumptive follicle (PO) which is attached to the nurse cell (NC). X 5 000. Both after Goldner stain.

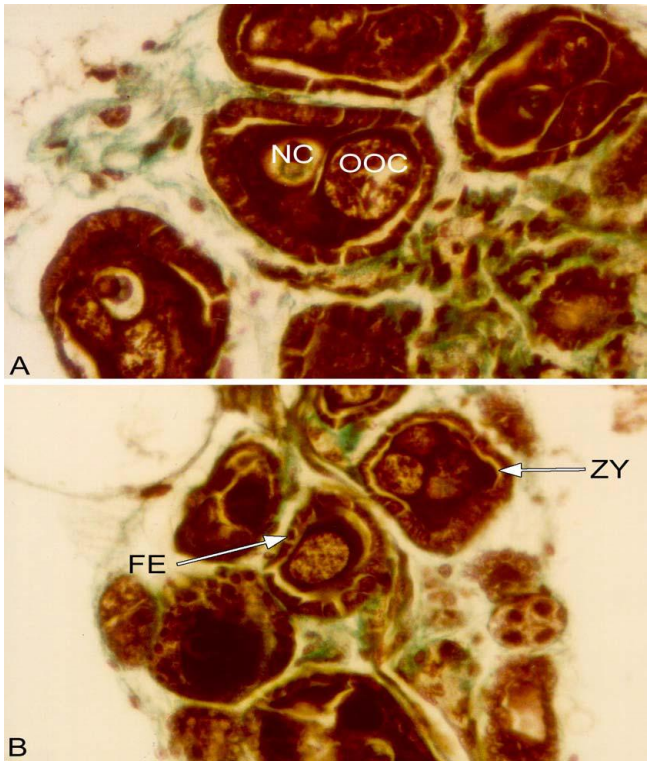

Fig 2: A. Light micrograph of an oocyte (OOC) and nurse cell (NC) of *Aedes aegypti*. X 10 000. B. Zone of yolk protein uptake (ZY) in the epithelial follicle (FE) in the oocyte of *Aedes aegypti*. X 7 5000. Both after Goldner stain.

Scanning electron microscopy

Scanning electron micrographs of ovarioles and Oocytes presented below were taken in order to understand the general structure of the mosquito ovaries (Fig.3A & B).

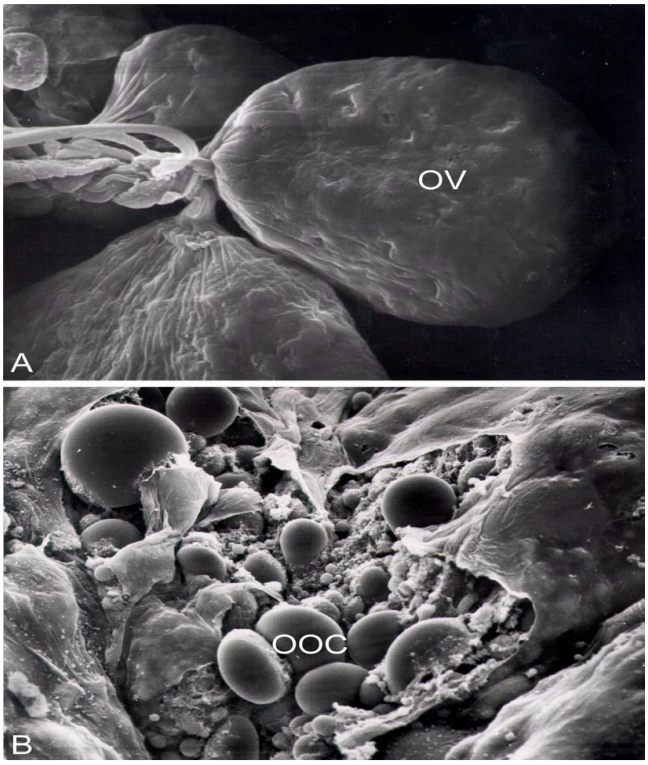

Fig. 3A: Scanning electron micrograph of the ovarioles (OV) of female *Ochlerotatus rusticus*. X 1 550. B.Oocytes (OOC) of female *Ochlerotatus rusticus*. X 1 050.

Discussion

A meal of blood or controlled diet leads to a series of changes in cell structures in the reproductive organ of female mosquitoes that quickly results to the formation of matured eggs. One of the prominent changes observed in the ovary of mosquitoes having blood meal is the presence of various unoriented microvilli in the area of the oocyte which is adjacent to the epithelial follicles (Fig. 2B). These microvilli can also be observed in ovary of mosquitoes having no blood meal but they are smaller in size. Roth and Porter (1964) [11] observed the same structural changes and they cited that the great numbers of microvilli are push into the extracellular spaces after 7 h of a blood meal. As it is depicted in this study, these microvilli are somewhat not uniform in lenght and are not regularly dispose as in typical intestinal epithelium which was described by Brandt (1958) [4].

Other prominent structural changes are observed in the region of the oocyte and epithelial follicles of the ovary. These changes are the occurrence of large intercellular spaces and the decrease in desmosomal connections in the epithelial follicle cells (Figs. 2A, B & 3-B). According to Roth & Porter (1964) [11], the epithelial follicle cells open channels between the extra-epithelial follicle space and the surface of the oocyte for the exchange of materials for the development of yolk.

Furthermore, pits or vesicular bodies are developed after a mosquito has taken a blood meal. Pits of similar structure obtained from this study have been reported in many cell types (Anderson, 1962; Roth & Porter, 1962; Roth & Porter, 1963) [10]. They are not to be confused with the simpler pits found especially in smooth muscle cells and blood vascular endothelial cells. The latter have simple, clean limiting membranes and are smaller in diameter (Palade & Siekevitz, 1956) [9].

The observations of pits in this study are in agreement with some other authors (Clements, 1992; Roth & Porter, 1964) [7,

[11]. These authors interpreted that these pits are engaged in pinocytosis which is associated in the uptake of materials for yolk formation. In the study done by Roth and Porter (1964) [11], it was found that the number of pits approximately 300,000 are observed in the oocyte after 7 h of a blood meal. This results in a 15 times increase of the number of pits found in the oocyte after the mosquito has taken a blood meal. Pits or vesicular bodies fuse to form small crystalline yolk droplets which subsequently coalesce to form large proteid bodies (Roth & Porter, 1964) [11]. Apparently, through the fusion of these various units of proteid bodies, the mature oocyte developed as depicted in this study in Figures 3A and 3B. The implication from the above observations and the literature references (Chargaff, 1942; Brambell & Hemmings, 1954; Smith, 1959; Christophers, 1961; Caro & van Tubergen, 1962) [6, 3, 13, 8, 5] on yolk synthesis is that these pits are responsible for taking up materials from the extracellular space of the follicle and contribute it to yolk granule formation.

Likewise, the results of scanning electron microscopy confirm the structure of the ovaries described by Clements (1992) [7]. The number of ovarioles ranges from 50 to 500 (Fig.3A &B) depending on the species, the physiological stages as well as the size of the individual female (Clements, 1992) [7]. Clusters of ovarioles are surrounded with a spongy fat body as depicted in this study by using light microscopy (Fig. 1A & B). This observation is in agreement with Roth and Porter (1964) [11] who described the yolk protein uptake in the oocyte of the mosquito *Aedes aegypti*.

There are reasons to believe that yolk deposition in the developing oocyte of a mosquito is accomplished by the removal of the protein from the blood (Smith, 1968) [14]. All of the structural mechanisms associated with rapid synthesis of proteins or lipoproteins, especially for segregation and storage in granules, appear in the fine structure of the epithelial follicles. Indeed, the very unsusual structural feature does appear, which is seemingly involved in the yolk deposition, are the development of pits or vesicular bodies (Clements, 1992) [7]. Generally, yolk develops rapidly, in fact, synthesis and storage are essentially completed at least 25 h after blood meal (Roth & Porter, 1964; Singh & Brown, 1957) [11]. By 4 h after the blood meal, these changes in the cell structure are already in evidence, and after 7 h they are very obvious (Roth & Porter, 1964) [11].

Conclusion

The present light and scanning microscopy play a valuable part in revealing some events of oogenesis in mosquitoes.

Acknowledgements

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