OOGENESIS IN THE BLACK-FLY, *SIMULIUM VITTATUM*

HISTOLOGY AND HISTOCHEMISTRY OF THE OVARY DURING OOGENESIS IN THE AUTOGENOUS BLACK-FLY *SIMULIUM VITTATUM* ZETT.

By

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Scope and Contents:

This research was designed to gain a better understanding of the oogenesis of the autogenous black-fly, Simulium vittatum and to compare it with studies on other Diptera. The autogenous character in S. vittatum showed only for the first gonadotrophic cycle. Since the cytoblasts are first produced in the late larval period, the late larvae, early pupae, late pupae and adults of various ages were prepared separately for histological and histochemical examination. The adult female flies were fed only sugar and water. The changes in size, histology, and histochemistry of the oocyte, follicular cells and nurse-cells during oogenesis were described and the possible physiological role of these cells in oogenesis suggested. The amount and composition of

larval fat-body remaining in females was compared with the stages of the oocyte development and the age of the flies.

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INTRODUCTION

General Aspects of Gametogenesis

The success of a living species depends upon its ability to reproduce and survive offsetting mortality produced by unfavourable environmental factors. Understanding gametogenesis is important in the appreciation of the success or failure of a species under certain conditions.

The first stage of gametogenesis is similar in both sexes involving cell proliferation by mitosis. The gametes then enter a period of growth. As the egg contributes the greater part of the substances used in nourishing the formation of a new organism, growth plays a much greater role in oogenesis than in spermatogenesis. Oogenesis is a combination of differentiation and growth. Phenomena occurring during the development of oocytes have occupied the attention of numerous investigators for many years (cf. Wilson 1937; Raven 1961). This interest reflects the important and basic position which the subject of germ-cell biology plays in any consideration of the life-history of organism.

In recent years the oogenesis of many insects has been studied (Popilius, Bryan 1954; Oncopeltus, Bonhag 1955; Tenebrio. Schlottman and Bonhag 1959, Aggarwal 1964; Periplaneta, Bonhag 1959, Aggarwal 1960, Anderson 1964; Hyalophora, King and Aggarwal 1965; Gerris, Eschenberg and Dunlap 1966; Pantala, Seshachar and Bagga 1963; Callosobruchus, Aggarwal 1967; Blattella, Amerson and Hays 1967). Some of the investigations have dealt with nematocerous Diptera (mosquitoes, Christophers 1911; black flies, Wanson 1948, Rubtzov 1955 and Madahar 1967) and others with cyclorrhaphous Diptera (Musca domestica L., Bier 1962, 1963, 1964; French and Hoopingarmer 1965; Goodman, Morrison and Davies 1968).

Several species of genus Drosophila have been studied and in these (melanogaster Mg., willistoni Sturt., pseudoobscura Frolowa, gibberosa Pat. & Main. and virilis Sturt.) the general sequence of events is the same (King and Wolfsberg 1957, Burnett and King 1962). For species of Drosophila, especially D. melanogaster, there is a larger body of information concerning oogenesis including the distribution of polysaccharides, lipids, protein and ribonucleic acids in developing oocytes(King, Rubinson and Smith 1956; King and Devine 1958; King 1960).

Autogeny of Diptera

It was long thought that bloodsucking Diptera needed a blood meal before their eggs reached maturation. Theobald (1901) was the first man to notice that some individuals of Culex pipiens L., now Culex pipiens var. molestus Forsk., can lay eggs without feeding on blood, and he coined the word autogeny to describe this phenomenon. Since then several autogenous mosquito forms have been found. Now a number of autogenous species of other blood-sucking Nematocera are known.

One general feature of autogeny is that one species may show this habit and a closely related species not. The variation may have a genetic basis which has been well investigated in the Culex pipiens complex (Rozeboom and Kitzmiller 1958), but in Diptera the difference in larval nutrition is also known to be important (Kardos 1959; Krishnamarthy and Laven 1961; Robin and Shortino 1962; Rubtzov 1955). Since autogeneous adults take no proteinous nutrient before developing eggs, Roubaud (1932) suggested that the mosquito larva stored up enough nutrient for later ovarian development in adult. Twohy and Rozeboom (1957) compared quantitatively the fat, glycogen and nitrogen composition of pupa and adult females of Culex molestus and C. pipiens reared under comparable conditions, and found significantly greater amounts of these materials in C. molestus. The poor larval

nutrition may reduce the number of eggs laid by autogenous mosquitoes (Gaschen 1932; Hecht 1933); it also affected all stages of growth of the oocyte (Laurence 1964).

Autogeny in the bloodsucking Simuliidae was first implied by Krafchick (1942) and Nicholson (1945), and was later substantiated and expanded (Rubtzov 1955, Davies and Peterson 1956, Downes et al. 1962). Nevertheless, most species require a blood meal for the first or at least subsequent gonadotrophic cycles.

Diptera, according to Downes (1958) and Corbet (1967), have evolved from bloodsucking ancestors and the acquisition of autogeny could be a more recent adaptive feature in evolution. But the mechanism by which these non-bloodsucking flies control ovarian development is still scarcely known. It may be true that some physiological factors are involved in stimulating autogenous ovarian growth. Since the females develop their ovaries at the expense of reserves carried over from the larval stage, it is quite understandable that the basic difference between the autogenous and anautogenous flies is the amount and type of reserve available to the adult.

The Ovary and the Fat-body with its Related Function

A certain amount of the food eaten by insects during larval life is stored in the cells of the fat-body. It is a store for lipid substances and is equally important for protein and glycogen. In female insects, most of theseare converted into the protein and lipid of the developing egg. Participation of the fat-body in oocyte growth in Drosophila was demonstrated in the paper by Doane (1963). Telfer (1963) indicated that very little fat-body remains in the female moth at the completion of yolk formation, while significant amounts remain in males at the same stage of metamorphosis. In females which have been ovariectomized as pupae, on the other hand, a substantial reservoir of fat-body tissue remains at the conclusion of metamorphosis. These results strongly suggest that yolk formation draws, to a major degree, on substances stored in the larval fat-body. Thus, a cyclical turnover of the stored components as ovarian development proceeds may be expected.

The two tissues will be discussed separately below.

1. Ovary

Each ovary consists of a number of ovarioles bounded by a simple epithelium. The number of ovarioles in each ovary is characteristic of the species.

Th riole is the functional unit of the ovary. In Diptera, it consists of parts - terminal filament, germarium and vitellarium (Harlow 1956; King and Devine 1958; Goodman et al. 1968). The wall of the germarium and vitellarium is formed of the tunica propria, outside which there is a cellular epithelial sheath being better developed in younger stages.

i. The terminal filament - is a solid strand of cells ensheathed in the tunica propria and the epithelial sheath; these form a suspensory ligament which may be anchored to the fat-body but generally is attached either to the body wall or dorsal diaphragm. In some insects the terminal filaments are separate from one another, and in a few cases they are absent.

ii. The germarium - contains densely packed germ cells in an active state of division and incipient differentiation. Germ cells soon develop into oogonia and the latter become differentiated into oocytes and nurse cells (trophocytes). In this area there are usually also smaller cells, which are of somatic origin and become the follicular epithelium in the lower part of the germarium.

iii. The vitellarium - enlarges rapidly as the oocytes multiply and mature, and varies in length according to the number and size of the eggs it contains. At the upper end of the vitellarium the follicle cells take a peripheral position and show the beginning of a

definite epithelial arrangement, while the oocyte and nurse cells assume an axial position. The growth of the oocytes divides the vitellarium into egg chambers, which become successively larger posteriorly. Beyond the last egg chamber, a mass of follicle cells forms a plug that closes the end of the ovariole.

According to Wigglesworth (1965), there are two types of ovarioles in insects: a) Panoistic type in which the nurse cells are wanting, and the yolk of the egg is formed solely by the follicular epithelium and b) Meroistic type in which the nurse cells are present to nourish the oocytes in the early stages of their development. The latter type is divided into two sub-groups: the polytrophic group in which each oocyte has a number of nurse cells enclosed with it in its follicle and the telotrophic group, in which the nurse cells are confined to the apex of each ovariole and are connected to the developing oocytes in the early stages of their development by means of long nutritive cords.

In Diptera, the ovarioles belong to the polytrophic type; the nurse cells arising from the primordial germ cells, are enclosed in the follicle with the developing oocyte. The number of nurse cells varies among Diptera, there are 15 in Simulium vittatum (Madahar 1967). These nurse cells plus the oocyte may arise from four consecutive synchronous cell divisions of an oogonium as is the case in Drosophila (King, Rubinson and Smith 1956) and Musca domestica (Goodman et al. 1968).

2. Fat-body

The fat-body is derived from the mesodermal walls of the coelomic cavities (Wigglesworth 1965). It loses its original segmental arrangement and forms a loose meshwork of lobes, invested in delicate connective tissue membrane, so that the maximum surface is exposed to the blood.

The primary function of the fat-body is the accumulation, storage, resynthesis of chemicals. It plays an important role in the processes of the insect, such as molting, metamorphosis, ovarian development, starvation and hibernation. It shows changes in relation to the different stages of development. According to Munson (1953), the fat-body cells of young larvae contain few inclusions, they build up reserves during larval life and the cells enlarge as they become loaded with fat, proteins and glycogen. During pupation there is some loss of reserves. At the approach of metamorphosis there is a change in the proportion and character of the stored reserves; the glycogen reserve built during larval life shows a decline although fat continues to increase for a longer period but then declines also (Munson, 1953).

The main value of the larval stages appears to be the accumulation of an adequate supply of all substrates which will be required for the histogenesis of the adult form. The nature of the reserves of the fat-body can be influenced by the type of food. It was

shown by Wigglesworth (1942) for Aedes aegypti L. that when the larvae were fed an adequate diet, the fat-body cells became loaded with fat, proteins and glycogen. If the starved larva was given carbohydrate alone the cells were stuffed with glycogen and there were relatively few fat droplets and vice versa (Wigglesworth 1965).

Glycogen is synthesized in the fat-body of the honey bee (Rockstein 1950). In some insects the metabolism towards fat synthesis is retained in adult stage (Gilmour 1965; Gilby 1965). Butterflies and moths continue to synthesize fat when fed only on sugar, as does the female mosquito, but male mosquitoes do not convert carbohydrate (glucose) into fat nor do male or female house-flies (van Handel and Lum 1961). Clements (1959) found that the locust fat-body can partially incorporate carbon atoms of amino acids into fat. The fat-body is one of the most active tissues in amino-group transfer (Gilmour 1965). Various types of metabolic activity are operative in the fat-body, enzyme systems are present for the synthesis to reserve materials from small molecules brought by the blood, and a good deal of inter-conversion is possible (Kilby 1963). The fat-body has often been compared with the liver of vertebrate: a general center of intermediary metabolism which serves also for the storage of reserves.

In the higher Diptera there is an almost complete dissolution of the larval fat-body, and a rebuilding of the imaginal fat-body. The disintegration of the larval fat-body may either occur during the latter part of the pupal period, or in the young adult, as is the case with muscid flies. The haemocoele of some newly hatched muscid flies is full of little floating spheres which are the remains of the larval fat-body, these are laden with protein granules, which disappear during egg development, they are then attacked by haemocytes (Wigglesworth 1965). However, at least in Musca domestica the larval fat-body cells are intact in the newly emerged female but quickly are depleted and atrophy during the first oogenesis (Goodman 1963). In the autogenous mosquito, after emergence the larval fat-body and certain of the larval abdominal muscles still remain within the adult. The abdomensof the newly emerged females of S. vittatum contain small eggs with a large fat-body (Davies and Peterson 1956).

Oogenesis and the Relationships between the Oocyte and the Auxiliary Cells

1. Oogenesis

This whole field has been reviewed in a most authoritative way by Raven (1961). Nevertheless, insect oogenesis provides many special characteristics (structures and processes), some of which have been reviewed by Bonhag (1958).

In polytrophic ovarioles, according to Raven (1961), the terminal region consists of a central mass of oogonia with large nuclei, and a peripheral layer of somatic cells with smaller nuclei from which the follicle cells take their origin. The oogonia multiply by division, and then give rise to oocytes and nurse cells.

Since the development of the black-fly ovary is similar to that of the fruit fly, Drosophila melanogaster, and house fly, Musca domestica; extensive use will be made of several recent articles on oogenesis in these insects. The developing egg of Drosophila melanogaster (King et al. 1956) and Musca domestica (Goodman et al. 1968) consists of a 16 cell nest, surrounded by an envelope of follicle cells. Fifteen of the daughter germ cells differentiate into nurse cells and nourish the most posterior daughter germ cell which becomes the oocyte. At first all 16 germ cells grow at roughly the same rate. However, once the vitellogenesis occurs, the oocyte grows much faster than previously partly at the expense of the nurse cells (King et al. 1956).

2. Nurse Cell

Nurse cells and the oocyte arise from the primitive sex cell, and there is an intercommunication of cytoplasm between members of these daughter cells. Concerning the intercommunication of cytoplasm, there are a few suggestions. From observations in Drosophila,

Meyer (1961) revealed that the cytoplasmic connection may, at some times, be occluded by a dense plug of homogeneous material, at other times the plug is perforated centrally and thus appears as a ring surrounding a zone of cytoplasmic continuity. He has shown that the plug serves as a valve which can control the timing of the interchange between the two cells and that a flow of material occurs when the plug is open. In a few cases, such as in Aedes, connections between nurse cells and the oocyte are missing during the period of yolk formation (Roth in Telfer 1965); when he observed vesicles of cytoplasm between the two cell types in Aedes, he was led to suggest that these are pinched off from the nurse cells and incorporated into the oocyte by membrane fusion. King and Devine (1958) found that the intercommunication of the cytoplasm among members of the 16-cell cyst in Drosophila is made possible by large pores in the membrane separating adjacent cells. Recently, Brown and King (1964) suggested that the simplest interpretation for cytoplasmic interconnections between the 16 cells of a developing egg chamber is that the ring canals mark the positions of the original oogonium. That is, a ring canal is formed as the result of an incomplete cytokinesis; where the centripetal advance of the cleavage furrow is stopped by the mitotic spindle.

Nutrient streamscan pass through several nurse cells, and eventually reach the oocyte (King 1960). Brown and King (1964) underthe electron microscope observed that mitochondria, small vacuoles, and strandsof endoplasmic reticulum passed through the cytoplasmic canal of Drosophila. Hsu (1952) suggested that in Drosophila mitochondria are transferred from the nurse cells to the oocyte and then aid the formation of the fatty yolk of the oocyte.

The nurse cells have as a rule a large, often branched nucleus. It is known from the work of J. J. Freed, summarized by Schultz (1956), that nurse-cell nuclei of Drosophila melanogaster undergo a series of endomitotic doublings of DNA and became polyploid.

There were some findings implicating nurse-cell nuclei in aiding the formation of yolky ooplasm:

1) King and Sang (1959) found that RNA synthesis occurs in the nurse-cell nucleus of Drosophila and that subsequently nuclear RNA enters the cytoplasm of the nurse cell and the ooplasm. Bier (1963) indicated the same sequence for Musca domestica. The contribution of RNA by trophocytes to the oocyte is known for two other species of insects with polytrophic ovaries, the silkworm (Columbo 1956) and the bee (Morgenthaler 1952; Bier 1954) and for two species with telotrophic ovaries, the bed-bug (Dasgupta and Ray 1954) and the large milkweek bug (Bonhag 1955). Although RNA that first accumulated in the ooplasm,

arose from the nurse-cell nuclei in Drosophila, King (1960) indicated that the subsequent build-up of ooplasmic RNA is independent of the nurse cells and the oocyte DNA. He explained that it may be that synthetic system originally located in the nurse cell nucleus is transferred to the ooplasm during vitellogenesis.

2) The rate of DNA synthesis in a nurse-cell nucleus reaches a maximum just prior to vitellogenesis (Jacob and Sirlin 1959).

3) As yolk formation begins there are bodies emitted from nurse-cell nuclei (Hsu 1953; King and Sang 1959, King and Mills 1962).

3. Follicle Cells

Mesodermal follicle cells which form an envelope about the cyst congregate in the posterior part of the germarium. The origin, division, migration, differentiation, and secretory behaviour of the follicle cells of D. melanogaster, as a function of developmental stage, have been described recently by King and Venoucek (1960).

It was long thought that, when nurse cells lose their secretory capacity, the follicular cells take over the task of nourishing the developing oocytes (Wigglesworth 1965). However, some workers disagree with this (Hsu 1952). Nonetheless, there is no doubt that the follicular cells play a part in mediating chemical substances passing to the oocyte from the haemolymph. As a rule, the passage of substances from the

follicular epithelium to the oocyte takes place in fluid form, and therefore is invisible and only indirectly demonstrable (Raven 1961). Raven (1961) presumed that the follicular cells may take up substances of low molecular weight from the environment, and synthesize them into higher compounds which are then transmitted to the oocyte. Although this is reasonable, it still needs to be proven. There were many reports (Bier 1962, 1963; Stay 1965; Ramamurty 1963; Roth and Porter 1964; Telfer 1961, 1963) showing that in insects the blood proteins were transported between the follicular cells into the oocyte. In polytrophic follicles of Culex, nurse cells do not appear to contribute to lipogenesis, so that the follicle cells are most important in the transport of lipids into the developing oocyte (Nath et al. 1958b). Glycogen occurs in the yolk of some insect eggs, normally it appears later in vitellogenesis after the trophocytes have degenerated; in these cases probably the follicle cells contribute the glycogen (Bier 1954).

The oocyte itself is contained in the vitelline membrane, which is believed by some authors (Beament 1946, Wigglesworth 1965), to consist simply of the modified plasma membrane of the oocyte. In Drosophila, however, evidence from the electron microscope makes it quite clear that the vitelline membrane forms as a condensation of droplets secreted by the follicle cells (King, Rubinson and Smith 1956, King and Devine 1958, King 1960) and this appears true for the housefly as well (Goodman et al. 1968). According to King and Koch (1963), the

vitelline membrane is formed by fusing numerous vitelline bodies within the intercellular space.

It is generally agreed that the chorion is also a product of the follicle cells. Chorion formation is initiated immediately after completion of the vitelline membrane (King and Devine 1958, Goodman et al. 1968). In Drosophila and Musca the chorion is first laid down upon the vitelline membrane as a thin sheet. Subsequently, the follicular epithelium pulls away from the inner sheet and lays down a second layer of chorion. The endochorion, which results, consists of an inner layer and an outer layer separated by a space (King and Devine 1958; Hinton 1969).

The follicular epithelium also serves in the resorption of yolk whenever an oocyte fails to complete its development in the vitellarium (Bonhag 1959).

Vitellogenesis

According to Raven (1961), the growth of the oocyte may be divided into two main periods. The first period is previtellogenesis (similar to "initial growth period" of Goodman et al. 1968) whichis characterized by minor augmentation of the cytoplasm. The second period is vitellogenesis which is marked by the appearance of the formed yolk,

a reserve of food substance, with which the developing embryo is equipped at the beginning of its career. Therefore, the synthesis and deposition of yolk constitutes one of the major events of oogenesis.

The precise manner by which vitellogenesis proceeds, depends to a considerable extent on which type of ovariole is involved. This has been reviewed by Bonhag (1958).

In the polytrophic ovariole, the developing oocyte with the nurse cells is enclosed in the follicular epithelium. There are many evidences that the nurse cells and the follicular epithelium play a part in the transfer of the nutrient substances to the oocyte. Hsu (1953) in his study of Drosophila melanogaster concluded that the protein yolk originated from nucleolar emissions of nurse cells. Therefore, the nurse cells and the follicular cells should not be overlooked in contributing to the synthetic activities associated with the yolk formation in the oocyte (Bonhag 1956; Bier 1963). Raven (1961) indicated that in general, the yolk substances are not ingested in finished form from the exterior, but a certain synthetic activity of the oocyte is involved in the establishment of their definite composition. In this synthesis of the yolk both the nucleus and the cytoplasm of oocyte with its inclusions may play a part.

Yolk is a morphological term rather than a definite chemical substance. It is deposited in the ooplasm in the form of globules or platelets. With regard to its chemical nature, three main components of the yolk may be distinguished: carbohydrate yolk, fatty yolk, and protein yolk. Due to the observations of Aggarwal (1962), Bonhag (1955), King (1960), Nath et al. (1958, 1959) with different staining, the yolk spheres have long been recognized as by far the richest depositories of protein in the oocyte and form a large part of the oocyte volume. The lipid yolk bodies are distributed among the protein bodies. There is histochemical evidence that free glycogen may not be present in the oocytes of all insects (Bonhag 1955). When present, glycogen particles can be found in the spaces between the protein bodies (Bonhag 1956).

During the course of oocyte growth almost all oocytes are involved in the production of protein or protein-carbohydrate yolk; although the extent or degree to which protein yolk deposition occurs relative to other kinds of yolk varies considerably among organisms or groups of organisms. While protein yolk deposition is almost a universal activity of oogenesis, it is true that the cytological mechanisms involved in this process are indeed varied.

The concept of the origin of yolk-protein is undergoing change. Earlier it was thought that the growing oocyte itself synthesized the yolk-protein. The large nucleus, in addition to the RNA-content of the ooplasm seem to favour this view. Raven (1961) indicated that the nucleus of the oocyte is the site of synthetic processes. King and Aggarwal (1965) in Hyalophora cecropia found that the nucleus of a stage-7 oocyte (ooplasm almost full of yolk) had a volume at least 300 times that found in stage 1 and is metabolically active. The nucleolus, which was known with a fair degree of certainty to play an important part in the synthetic processes occurring in the nucleus, displays a great activity especially during oogenesis. The extrusion of nucleolar material into the cytoplasm during oogenesis has been reviewed by Bonhag (1958). In many cases therefore it can hardly be doubted that it is of general occurrence, and it was believed by some investigators that nucleolar emissions from the nucleus play a part in the production of protein yolk (Bonhag 1958).

Kessel and Beams (1963) indicated that in the sea cucumber, Thyone briareus, once the nucleolar material has been transferred to the cytoplasm, it appears to break down into smaller masses and even individual particles. A portion of them may give rise to the large number of ribosomes which are scattered throughout the ooplasm. With the

electron microscope, they showed that the considerable amounts of ribonucleoprotein are transferred from the nucleus to the ooplasm.

Yolk protein of the egg of the domestic fowl, chemically related to the serum proteins of the laying hen, was first suggested by Jukes and Kay in 1932. Since then, several investigators have confirmed this finding. The first conclusive evidence that blood proteins are incorporated by arthropod oocytes emerged from Wigglesworth's spectroscopic analysis of bloodsucking insects and arachnids in 1943. Telfer (1954) by immunological procedures showed that in saturniid moths most, and possibly all, proteins present in the blood at the time of yolk formation are also detectable in the yolk. Telfer (1960), as in the experiments of Knight and Schechtman (1954), reported that even foreign proteins which have been injected into the blood are detectable in the yolk. Telfer (1961) indicated that blood proteins which have entered the moth oocyte are laid away in discrete cytoplasmic particles, the protein yolk spheres. These observations on oogenesis in the vertebrates as well as Telfer's work with the saturniid moth demonstrated conclusively that, in many instances of egg protein production and deposition, the protein species is made outside of the ovary, probably in the liver or an analogous organ, is secreted into the extra-cellular spaces, and is then removed from the circulating blood to the developing egg.

A number of investigations, when considered together, provide evidence that in certain animals precursors of yolk protein are manufactured outside of the ovary carried via the circulating blood to the follicle, transported by means of micropinocytotic vesicles into the oocyte cytoplasm and finally, perhaps utilizing synthetic machinery of the oocyte itself, become organized into a fully formed yolk granule or platelet (Panje and Kessel 1968).

In insects, it seems that much of the protein yolk appears to be incorporated into the oocyte directly from the hemolymph and subsequently organized into yolk bodies (Roth and Porter 1962; 1964; Kessel and Beams 1963; Anderson 1964, Stay 1965).

In crayfish, it was found that endoplasmic reticulum of the oocyte synthesizes the yolk protein (Beams and Kessel 1963; Kessel 1968).

In the tunicate, Ciona intestinalis, Kessel (1966) found that materialsincorporated into the oocyte in micropinocytotic vesicles were added to the forming yolk granules during the final stages of their maturation.

As a result of the afore-mentioned investigations, Kessel (1968) pointed out that during the process of vitellogenesis, the possible origin of yolk formation of most animals can be included into the following processes: a) extraoocyte synthesis of yolk, b) intraoocyte synthesis of yolk and c) a combination of both activities.

The Route of Entry of Extraovarial Yolk Protein

Since it appears that in a number of insects yolk proteins come from an extraovarian source, how the blood proteins might be transferred from the blood to the growing yolk spheres is discussed below.

The first barrier which must be traversed is the epithelial sheath. The follicle in an ovariole is continuously surrounded by the epithelial sheath and tunica propria. The individual oocyte is then directly enveloped by a single layer of follicle cells whose outer surface adheres to the inner side of the tunica propria. In some insects, the vitelline membrane is laid between the oocyte surface and the follicle cells before yolk formation is completed (Bier 1963; King and Koch 1963).

The epithelial sheath becomes attenuated during yolk formation so that its structure is difficult to determine by light microscope in sectioned material (Telfer 1965). Bonhag and Arnold (1961) observed that in Periplaneta americana (L.) it consists of an open meshwork of tracheolar cells, mycetocytes, and an additional component akin to the cells of the fat-body. In cecropia moth, an analogous meshwork consists of tracheolar cells and striated muscle fibers (Telfer 1963). Aedes aegypti revealed a loose system of striated muscle fibers (Roth and Porter 1964).

Telfer (1965) indicated that the tunica propria which is about 1-2μ thick could reduce the rate of diffusion of protein from the blood into the egg but he also indicated that proteins penetrate it fairly readily in both directions. Bonhag and Arnold (1961) suggested that the tunica propria has the potential of serving as a selective dialytic membrane regulating chemical exchanges between the developing egg and haemolymph.

It was noted by Telfer (1961) that the follicle cells do not form a continuous layer of cells in the cecropia moth; during yolk formation there are substantial intercellular spaces between them. Intercellular spaces, running from the tunica propria to the oocyte surface through the follicular epithelium, have also been described in Calliphora and Musca (Bier 1963), Panorpa (Bier 1964) and Aedes (Roth and Porter 1964). Concurrent with the formation of the inter-follicular cell spaces, the follicle cells in many cases lift from the surface of the oocyte, remaining in contact with both the oocyte and with each other by long microvillar processes (Telfer 1965). According to Telfer (1963, 1965) and Stay (1965) extraovarian proteins reach the oocyte surface mainly by passing through inter-follicular cell spaces. King and Aggarwal (1965) indicated that blood protein can also enter the follicle cell in pinocytotic vesicles which are immediately broken down to amino acid, so they were not retained in high enough concentration in Telfer's sections to be detected.

The vitelline and tunica propria of a number of insects are permeable to molecules with molecular weights of several hundred thousand (Telfer 1965). At present it is considered that the function of the tunica propria is to maintain the morphological integrity of the ovariole.

After passing through the above mentioned barriers, the blood proteins must then be transported in some manner into the growing yolk spheres. Ramamurty (1963) indicated that the blood proteins were not brought into the oocyte by the increasing permeability of the oocyte membrane. Roth and Porter (1962) and Kessel and Beams (1963) concluded that the actual uptake of the molecules into the ooplasm takes place through a process of micropinocytosis. The mechanism suggested is based on an analogy with what is known about protein uptake from the environment by amoebae.

Pinocytosis

Lewis (1931), from observations on macrophages and sarcoma cells in tissue culture first defined pinocytosis as the uptake of fluid droplets by cells.

It is a mechanism by which cells transport solutes across the surface membrane (Brandt 1958). According to De Robertis, Nowinski and Saez (1965), pinocytosis is not an alternative process to active transport, but rather a supporting one. By means of pinocytosis the cell is provided with a much larger interior interface where passive and active

transport are carried out more efficiently than at the surface membrane. Brandt (1958) found that all solutions of charged molecules, including many protein and salts, would induce pinocytosis and their activity was dependent to some extent on their concentration, but Rustad (1961) indicated that carbohydrate would not. He also indicated that CO and cyanide caused pinocytosis to disappear. These inhibit the formation of ATP. So apparently pinocytosis requires ATP and therefore involves at least one energy consuming chemical reaction.

The processes of pinocytosis consist of two steps (Rustad 1961). The protein is first bound to the outside of the cell membrane and is then "swallowed" as pinocytosis channels form and are pinched off. The phenomenon of binding suggests that the cells have specific chemical groups in their membrane surface that selectively take up certain molecules.

Telfer (1963) noticed that pinocytosis had several points of similarity with yolk formation by the moth oocyte. This mechanism was also indicated in scorpionfly Panorpa communis (Ramamurty 1963), milkweed bug Lygaeus kalmii (Kessel and Beams 1963), Periplaneta americana (Anderson 1964), Gryllus bimaculatus (Favard-Sèrèno 1964) and Aedes aegypti (Roth and Porter 1964). The surface of an amoeba undergoing pinocytosis has a ruffled appearance and is like the brush border of the oocyte. The protein, having entered the cell, becomes

associated with a cytoplasmic membrane first then is liberated into the cytoplasm. This binding feature of the membrane suggests a mechanism which could underlie the selectivity of blood-protein uptake in the oocyte. This agrees with Telfer (1960) and the "female" protein is the one most avidly accumulated in oocyte, while the non-insectan proteins which were injected into haemolymph are detectable in the oocyte in relatively small amounts. This may result from the avidity of the female protein for a surface-adsorbant on the oocyte, similar to the mucoid coat on the surface of an amoeba.

The Selection of the Problem

Black flies (Simuliidae) occur throughout the world and the females of most species pierce the skin and suck the blood of mammals and/or birds. Being responsible for the spread of human onchocerciases in Africa, Mexico, Central and South America (Wanson 1950; Dalmat 1955; Crisp 1956) as well as the irritation and the toxic and allergic manifestations of their bite, they have great economic importance in many parts of the world. Nonetheless, until now there have been few studies on oogenesis in blackflies. Puri (1925) measured egg size in several European species. Wanson (1948) was first to describe the gonotrophic cycle of the African Simulium damnosum Theob. Davies

and Peterson (1956) indicated that the eggs of some Ontario blackfly species are mature, or almost mature when the female emerges from the pupa and in other species the females had eggs in various stages of development and also various amounts of stored nutrient.

Simulium vittatum Zett. is one of the most abundant and widely distributed species in the province of Ontario and is multivoltine (Davies, Peterson and Wood 1962). It is 100% autogeneous for the first cycle, while a blood meal is needed for subsequent cycles (Davies, personal communication). Although the gross histological aspects of egg development in this species were determined by Madahar (1967), he did not study the histochemical changes in the ovary during oogenesis.

The present study examines in more detail the cytological and the histochemical changes in the ovary of Simulium vittatum during the autogenous first gonadotrophic cycle.

MATERIALS AND METHODS

1. Collection and Maintenance of Experimental Flies

Maturing larvae of Simulium vittatum were collected in Redhill Creek at Albion Falls, Hamilton, Ontario, in the late summer of 1968. The larvae for sectioning were kept in petri dishes on filter paper well moistened and maintained in a refrigerator at 10°C for 1-2 days. The filter paper was changed a few times to make sure that the larval digestive tract was clean, otherwise the contents would have interfered with the sectioning.

Some of the adult blackflies used were reared from pupae collected in Spencer Creek at Tew's Falls, Dundas, Ontario, in the early summer of 1968 and others from larvae and pupae collected at Albion Falls. The larvae were reared in the laboratory at 13-15°C in plastic containers (7.5 x 7.5 inch) which were half full of water agitated by air bubbles. A few drops of bakers' yeast were added each day as food. When too much yeast was added, the water became unclear and larvae were less successful. Pupae from laboratory-reared larvae and those collected from the field were separated by sex, if possible, and kept in petri dishes on filter paper well moistened with distilled

water. Some of these pupae were prepared for study. The rest of them were maintained in a refrigerator at 10°C and checked for emergence every half day. When pupae were kept at room temperature the time to adult emergence was short but the pupal mortality was increased consequently.

Emerged adult females were sorted and maintained at 13-15°C. in cardboard cylinders (3.4 x 4.5 inch). The top and the bottom of the cylinders were covered with nylon screen. The bottom of the cylinder was put on water-soaked absorbent cotton in a plastic Petri dish. The adults were supplied only with sugar cubes on the top screen of the cylinder. The females studied were all virgin, since mating had been unsuccessful in small cages (Madhar 1967).

2. Preparation of Sections

Aspects of oocyte differentiation and all of the histological changes were studied in fixed, sectioned and stained preparations of whole flies or whole abdomens using cytochemical techniques and the general light microscope. At least ten females were selected at random at certain intervals after emergence, so that some individual variability could be appreciated. This was because females, which were selected, had emerged over a period of 5-10 hrs.

Before fixing, the flies were stunned with chloroform. Because of chitin, penetration of fixatives and sectioning of insects can be difficult. Thus, as soon as the flies were stunned with chloroform, the legs and wings were quickly removed with forceps. Although it is easier to do this after the flies are kept in Bouin's fixative for a few hours, the presence of the legs and wings makes flies float on the fixative and interferes with the penetration. When many flies were to be fixed at one time, most of them were kept refrigerated at 35-45°F until used.

Whole flies or abdomens were placed in Bouin's fixative or Bouin's fixative plus dioxane (1:1); the latter solution was more successful in fixing the yolk (Rugh 1948). They were left in the fixatives for 48 hrs. or longer, as several weeks in these solutions causes no damage. Each tissue was washed in 70% alcohol with daily changes until the yellow colour ceased to appear in the washing alcohol. The yellow colour must disappear before sections are stained. It was usually removed in the alcohol series, but, if not, slides were treated in 70% alcohol plus a few drops of saturated lithium carbonate until the colour was extracted. After being washed in 70% alcohol, the tissue was passed through 80% and 90% alcohol (1 hr. in each solution), and then through two changes of absolute alcohol (1-2 hrs.) to ensure complete removal of water.

The dehydrated material was cleared in cedar-wood oil. Usually overnight was required for complete replacement alcohol in the tissue. When the tissue was incompletely dehydrated, the cedar-wood oil became solidified during the clearing process. The only remedy was to return the tissue to absolute alcohol to eliminate the water and then to place it again in a fresh supply of the clearant. Embedded tissue containing water can shrink, and this offers difficulties in sectioning and mounting sections on slides. The cleared tissue was transferred directly from the clearant to melted Tissuemat (m.p. 56°C) already prepared in an oven, two changes of 1.2. hrs each. The oven temperature should be kept just high enough to maintain the paraffin in a melted state. This lessens the danger of overheated tissue, which can initiate hardness and shrinkage. Paraffin standing in a warm oven in a melted condition for some time is better for infiltrating and embedding than freshly melted paraffin.

As soon as the tissue was thoroughly infiltrated with Tissuemat, it was ready to be embedded. The tissue was oriented in a small container already filled with melted Tissuemat. When a solidified skin had formed over the block, it was cooled immediately in water, preferably at a temperature of 10-15°C, since water colder than 10°C caused the block to contract too strongly and finally crack.

The blocks were trimmed, and sections 7-10μ in thickness were cut using a AO Spencer Rotary Microtome. Sections were transferred directly with a camel's hair brush to water drops on albuminized microscope slides. When the sections were spread smoothly, the excess water was drained off. Then the slides were left on a slide warmer until the water had evaporated and the sections were firmly attached to the slides.

3. Histochemical Methods

Various histochemical tests were applied to the granule-filled follicle in an attempt to determine the nature and composition of follicle. For histochemical analysis the following tests were performed on Bouin or Bouin-dioxane fixed materials.

a) Delafield's hematoxylin-eosin stain (Humason 1967) was used to determine the general histological changes. In this case the nuclei showed deep blue, while cytoplasmic structures were rose coloured.

b) Mercuric bromphenol blue (Mazia et al. 1953) and the acidic dye of fast green (pH 1.2, King 1960) were used as general protein stains. In the mercuric bromphenol blue method, protein and peptides appear blue. The acidic dye, fast green, forms compounds with positively charged molecules. Under the acidic conditions employed, almost all the groups capable of being positively charged in all the proteins are ionized, and consequently, according to King (1960), all proteins should

bind the dyes, they show green. The stains were brought to the desired pH with 0.1 N HCl.

c) Sudan black B (McManus 1946 in Pearse 1961) and oil red O (Humason 1967) were used for general lipid staining in paraffin sections. Sudan black B is considered the most sensitive of the lipid dyes. A saturated solution of Sudan black B in 70% alcohol was made. In this method saturation is essential. There must be excess dye, and several days must be allowed to ensure complete solution. Longer times of staining are advisable. Lipids, if present in sufficient quantity, stain black or blue in Sudan black B, and show orange or brilliant red in oil red O.

d) Periodic acid-Schiff staining was utilized for the detection of polysaccharides. Since diastase can remove glycogen, the identity of glycogen in PAS-positive material was investigated by treating for at least 3 hours at 37°C one or more sections of the same series (before staining) with a 1% solution of Taka-diastase (Park, Davis and Co. Ltd., Brockville, Ontario), containing 1% NaCl. The presence of glycogen was also determined directly by means of the Best's carmine technique, a positive reaction showing a red colour.

e) Spicer's Feulgen methylene blue stain was used for nucleic acids (Lillie 1965). The stain in pH 3 or 3.5 McIlvaine citric acid disodium phosphate was used. The lower pH level gives less dense thiazin staining. Ribonucleic acid appears blue.

The preparations were completed by covering the sections with Permount, or for lipid-stained material with glycerol jelly, and by adding a coverslip.

4. Measurement of Follicles from Histological Sections

Using an ocular micrometer, measurements were made of sections prepared by Bouin's and stained with Delafield's hematoxylin-eosin. The method was based on that used by Goodman, Morrison and Davies (1968). The diameter (d) and length (l) of the nurse cells, nurse-cell nuclei, oocyte, oocyte nucleus, follicular cells, as well as the follicle as a whole, were measured. From these measurements the approximate volumes of the nurse cells and their nuclei, the oocyte and its nucleus and the follicle are calculated, using

$$v = \left(\frac{\pi}{\ell}\right) d^2 \, l$$

In making these measurements, only follicles in which the section passed through, or close to, the major axis were used. In such sections the follicular cells appeared as a single layer. About two to four nurse cells were measured from each follicle, in such a way that one to two were adjacent to the oocyte, one to two were intermediate, and one to two were distal to the oocyte. From these measurements the average volume per nurse cell was calculated, which, if multiplied by 15, should give the total contribution of the nurse cells to the volume of the follicle.

RESULTS

Cytological Changes of the Ovary

Introduction

Development of the follicles of the autogenous Simulium vittatum, started before emergence, so that when the adult female emerged from the pupae, the eggs were about half grown. Because of this, it was impossible to study the early stages of oogenesis in the adult fly for the first gonadotrophic cycle. Also, no one has been able to make this species feed on mammals in the laboratory so that it was out of the question to study subsequent gonadotrophic cycles. This is quite different from the case in Drosophila (King et al. 1956) and Musca domestica (Goodman et al. 1968), where several cycles can be promoted in the laboratory, making it possible to study all stages of oogenesis in the adult fly.

The oogenesis of S. vittatum began in the late larval stage. Therefore, for a complete sequence of oogenesis, it was necessary to go back to the larva and follow ovarian development in the pupal and adult stages. Accordingly, the description of histological changes of ovary during oogenesis in S. vittatum is divided into the following periods:

1. The late larval period
2. The early pupal period
3. The late pupal period
4. The adult period

The dimensions given in the following account refer to those measured in paraffin-embedded material. The formula of

$$\sqrt{\frac{\Sigma X^2}{n} - \left(\frac{\Sigma X}{n}\right)^2}$$

was used for calculating standard deviation. The 14 stages, assigned by King et al. (1956) to the developing Drosophila follicle, were used in this study. The term "stage", when considering growth, refers to a range in size.

The Late Larval Period

The ovaries lay on each side of the intestine in the posterior abdomen. When examined under the light microscope, each appeared as an elongated mass of cells, tapering anteriorly into a filament which ended in the fat-body (Fig. 12). The whole ovary was enclosed by an epithelial sheath of adventitious connective tissue. The differentiation of ovarioles had already begun during this period. Inside the ovariole there were groups of mitotically dividing cells. Among these groups there were single cells and clusters of 2, 4, 8 and 16 cells. The

single cells were always the largest, resided at the anterior end of the germarium and were presumably oogonia. The more posterior germarial cells were smaller and in clusters which finally reached a total of 16 cells. Each group of 16 cells, presumably arose from a single oogonium by four consecutive and synchronous divisions, and it seemed that with each division the cells became smaller and smaller. The morphology of the nucleoli of the nurse cells and oocyte were the same at this time. Whether these cells were interconnected by cytoplasmic bridges was not demonstrable with the technique used in these studies. During this period the most prominent part of the cell was the nucleus which occupied almost the whole cell. As the groups reached 16 cells, they were eventually surrounded by a layer of mesodermal cells, the follicular epithelium (Fig. 1). This is the beginning of the egg chamber. Before pupation started the posterior end of germarium was full of these egg chambers each of which was spherical about 1.8μ in diameter. The oocyte was difficult to distinguish from the nurse cells since it grew at an identical rate to that of the nurse cells.

The Early Pupal Period

The follicle increased in size because of the enlarging of the 15 nurse cells and oocyte. Pupae fixed just after pupation had stages of egg development identical to those found in the late larvae

about to pupate. In early pupae the egg chamber, which was spherical, was separated from the germarium by the interfollicular tissue that formed a stalk (Fig. 13). At this time each germarium contained a germarial cyst, and each vitellarium one egg chamber. In morphology these egg chambers corresponded to stage 3. Little nuclear structure of the oocyte and nurse cells was distinguishable (Fig. 2A), but presumably the most posterior one was the oocyte (King et al. 1956). In nurse cells the ratio of cytoplasm to nucleus began to increase and at stage 5 it was around 2:1 (Table 1 and Fig. 9). The follicular epithelium increased in height over both oocyte and nurse cells; at stage 5 the follicular epithelium surrounding both oocyte and nurse cells was 1.8μ (Table 1 and Fig. 10). The nucleus of the oocyte and nurse cells underwent morphological changes, but this differed between oocyte and nurse cells. The follicles in all the ovarioles were at the same stage of development and thus also comparable in size.

The Late Pupal Period

In late pupae the vitellarium contained an advanced follicle (about stage 6-8). The follicle grew and elongated in the vitellarium during this period. The oocyte grew more rapidly than the average nurse cell. At stage 8 the cells of the follicular epithelium already

exhibited regional variations in their structure becoming cuboidal (2.4±0.0μ) over the nurse cells but columnar (3.3±0.3μ) over the oocyte (Table 1 and Fig. 10). The nurse cells could now be easily distinguished from the oocyte on the basis of nuclear morphology, because the nurse cell nuclei had multiple nucleoli, whereas each oocyte nucleus possessed a single nucleolus (Fig. 2B). The round oocyte nucleus (about 10μ in diameter) lay in the center of ooplasm, and contained a spherical nucleolus nearly 5μ in diameter. The ratio of nurse cell cytoplasmic volume to nucleus remained at 2:1 (Table 1 and Fig. 9) as in the larval stage. The nurse cells and their nuclei increased in volume simultaneously during this period (Fig. 9). In a given follicle, however, the nuclei of the 15 nurse cells differed in size. The nurse cells closer to the oocyte and to the follicular epithelium were larger as were their nuclei, while the nurse cells in the middle seemed smaller. Just before the adult began to darken prior to emergence from the pupal skin, the oocyte was distinctly larger than the average nurse cell (Fig. 2B).

Table I

Size of the follicle, the oocyte and the nurse cells; and the height of the follicular epithelium over the oocyte and over the nurse cells as related to the stage of development of the follicle

Stage	Volume ($\mu^3 \times 10^{-3}$)					Height (μ) of follicular cells over		Diameter (μ) of oocyte
	Follicle	Oocyte		Nurse cells		Nurse cells	Oocyte	
		Total	Nucleus	Total	Nucleus			
1	2.938±0.046					0.94±0.3	0.94±0.3	
2								
3	3.538±0.00					1.2 ±0.0	1.2 ±0.0	
4								
5	6.912±0.00	0.303±0.026	0.064±0.011			1.8 ±0.0	1.8 ±0.0	10.33±1.248
6	9.046±0.075	0.432±0.041	0.117±0.015	4.992±0.948	1.404±0.197	2.04±0.29	2.04±0.29	11.50±0.870
7								
8	22.18±3.509	1.996±0.215	0.369±0.073	11.79±1.656	4.627±1.591	2.4 ±0.0	3.3 ±0.3	18.40±0.8
9	50.75±14.19	10.84±3.349	1.132±0.259	16.87±3.186	5.501±1.986	1.8 ±0.15	4.49±0.06	29.85±5.105
10	103.4±22.32	36.62±14.88	3.502±1.190	23.88±0.611	10.85±1.668	1 ±0.18	5.58±0.38	42.28±8.070
11	402.7±66.06	258.8±88.84	8.699±1.390	20.22±4.807	7.080±1.179		4.73±0.71	69.41±14.41
12	733.2±131.6	517.8±95.89	12.29±1.878				3.84±0.54	87.42±7.150
13	857.8±139.1	701.6±135.8	14.26±2.702				2.33±0.09	93.90±10.30
14	956.7±13.71	826.4±112.6					1.03±0.23	97.50±4.930

The Adult Period

Cytological changes were evident during the period from the time of emergence of the fly to the maturity of the egg, such as the appearance of yolk spheres in the oocyte, the increase in the size of the oocyte due to the accumulation of yolk (Fig. 7 and 8), the changes in the oocyte nucleus (Fig. 7), the changes of the nurse cells (Fig. 9) and follicular epithelium (Figs. 4 and 10), and the formation of the vitelline membrane and chorion (Fig. 4).

In newly emerged adult females of S. vittatum, each ovariole was enclosed in a thin, muscular epithelial sheath which became indistinguishable in older flies. Each ovariole included a posterior, larger follicle; one or two presumptive follicles; and the germarium (Fig. 3). At this time the oocyte was markedly larger than the average nurse cell, it comprised about half of the total volume of the follicle which was ellipsoidal (Fig. 14). The oocyte nucleus was prominent in the adult period and always lay in the center of the oocyte. The spherical nucleus, although smooth at first developed a little irregular outline with a slightly folded membrane in the later stages. Around the nucleus of oocyte at stage 9, there was a thin layer of perinuclear granules which were blue in hematoxylin eosin stained sections (Fig. 15). The follicular epithelium around the nurse cells had changed from a cuboidal to a thin squamous layer, whereas those around the oocyte had become

taller in height (4.45±0.06μ) (Table 1 and Fig. 10). The number of follicular cells over nurse cell was less than those over oocyte per unit area. The ratio of nurse cell cytoplasm to nucleus remained almost constant during adult period (2:1) (Table 1 and Fig. 9). Yolk deposition took place mainly during late stage 9 and 10. The yolk spheres were generally circular in profile. When the yolk grew actively during stage 10, the perinuclear area became prominent, being full of blue granules. At stage 10, the oocyte began to elongate; the nurse cells with their nuclei had reached their maximum volume and thereafter stopped growing (Table 1 and Fig. 9) and the follicular epithelium reached its maximum height (5.58±0.38μ) (Table 1 and Fig. 10) and became columnar. During this stage, the follicular cells started secreting the vitelline membrane; at first there were small spheres (Fig. 4) which fused together and became a thin layer. At late stage 10 the follicular epithelium around the oocyte decreased in height becoming cuboidal once more (4.73±0.71μ) (Table 1 and Fig. 10), the nurse cells shrank slightly, and gradually were compressed into a narrow, anterior disc by the enlarging oocyte. At stage 11, the vitelline membrane was completed (Fig. 4), and the secretion of the chorion began between the follicular epithelium and the vitelline membrane. During stage 12, the follicular epithelium of the oocyte became low

cuboidal (3.84±0.54μ) (Table 1 and Fig. 10) and by stage 13, the chorion was completed (Fig. 4 and Fig. 16) and the nurse cells which remained outside the chorion on one side of the egg occupied only a small fraction of their former volume. During the growth of the oocyte the nucleus continued to increase till stage 13 (Fig. 7), though not to the same extent as the cytoplasm; then its nuclear membrane disappeared in late stage 13 before egg maturation. Each oocyte nucleus contained a large nucleolus lying in the center of the nucleus. The nucleolus often appeared as spherical, but occasionally it developed a horse-shoe shape (Fig. 17). In the early oocyte, the nucleolus was homogeneously stained, but in later stages there were differences in stainability (Fig. 18). The diameter ratio of oocyte nucleus to nucleolus was around 3:2 or more in the adult period as in the pupal stage. The follicular epithelium around the oocyte became squamous during stages 13 and 14 (1.03±0.23μ) (Table 1 and Fig. 10).

Rate of Oogenesis

The oogenesis of S. vittatum began in the late larval stage. Newly formed pupae contained follicles at stage 3. The oocyte grew at an identical rate to that of the nurse cells during the early pupal stage whereas in the late pupae, the oocyte grew faster. By this time the

follicles had developed to stage 8 and the volume of the follicle was about six times more than in the early pupal period (Table 1 and Fig. 6). Follicles grew slowly at first then increased much more rapidly later in the pupal stage. Most of the increment in volume resulted from an increase in the size of the 15 nurse cells, although the oocyte was growing more rapidly than the average nurse cell in the late pupa. According to Madahar (1967), the pupal stage lasts about eight days at 5-6°C. When the fly emerged from the pupa, the follicle of each ovariole had already developed to stage 9 (Fig. 11), and it had increased 2.5 times since the late pupal period (Table 1 and Fig. 6). It took almost 5-6 days for eggs to become mature (stage 14) at 13-15°C. (Fig. 11). The volume of the follicle obviously increased in the adult period (Fig. 6), the greatest development being between the time of adult emergence and the end of the second day when a ten-fold increase occurred (Table 1 and Fig. 6). During this period the majority of growth of the oocyte took place as well (Figs. 7 and 8). This generally coincided with the time of vitellogenesis. Although the vitelline membrane was completed around stage 11, about 2 days after emergence (Fig. 11), the follicle as a whole still increased, but at a reduced rate (Fig. 6). In a given fly, the development of the follicle of each ovariole was regularly at the same rate. But the flies at the same age showed different stages of ovarian development (Fig. 11). In "old" flies, two days after emergence

(around stage 11-14), there were sometimes a few oocytes in the ovarioles that failed to reach maturation and were resorbed by the follicular epithelium. Resorption of oocytes seemed not uncommon in S. vittatum under the laboratory conditions. During this process the follicular epithelium became very thickened and vacuolate, and the nuclei of the follicular epithelium lost their precise arrangement (Fig. 19).

Histochemistry of the Developing Follicle

Carbohydrate

In early stages of oogenesis the whole structure of the follicle was PAS negative. The first PAS-positive bodies appeared by stage 10 in the peripheral ooplasm adjacent to the follicular epithelium (Fig. 20). These bodies increased in both size and number, and also became more centrally distributed. Around the end of stage 10 the whole ooplasm was full of PAS-positive spheres (Fig. 21). These yolk spheres were positive to PAS until the end of oogenesis. In order to determine if the PAS-positive reaction in yolk spheres was due to glycogen, sections of the same series were treated for 3-12 hr. with a 1% solution of Taka-diastase containing 1% NaCl. These PAS-positive bodies were diastase-resistant, as they appeared PAS-positive both before and after treatment. Thus, the PAS-positive reaction was not due to glycogen. That was improved by Best's carmine technique. To test whether carbohydrate - protein complexes were responsible for

the PAS-positive reaction, the fast green (pH 1.2) method for protein (King 1960) was employed. The yolk spheres gave an intense coloration with fast green, indicating that these sites were strongly protein positive.

No PAS-positive bodies could be detected histochemically in the follicular epithelium nor in the nurse cells at any stage of oogenesis.

Protein

According to King (1960), fast green at pH 1.2 would indicate the general distribution of protein in the ovarioles. The nucleus and cytoplasm of the nurse cells, follicle cells and the oocyte gave a positive reaction with mercuric bromphenol blue and with fast green (pH 1.2), the latter giving better results. The amount of fast green bound by the above mentioned structures varied little in the different stages of developing follicle. These structures reacted positively to fast green at pH 1.2, both before and after extraction with hot trichloroacetic acid. In the cytoplasm of the oocyte the protein occurred in two places: mainly in the yolk spheres and a certain amount in the background ooplasm. About stage 10, the follicular cells stained more intensely with fast green than the oocyte, which meant that they contained more protein.

Lipid

Employment of Sudan black B and oil red O stains, specific for lipids, showed that lipids as small granules were present in all stages of follicles. When comparing the nurse cells, follicular cells and oocyte, the last contained the most lipid. In the early oocyte, before vitellogenesis, the lipid bodies were dispersed through the ooplasm (Fig. 5A). As the oocyte grew, lipid bodies became more prominent. At the end of stage 9, there were many small lipid granules, especially in the cortex near the follicular epithelium. During and after yolk formation, the lipid bodies appeared as granules well dispersed in the ooplasm among the carbohydrate-protein yolk spheres (Fig. 5B).

Ribonucleic Acid

The cytoplasm of the nurse cells, oocyte and follicular epithelium was positive for RNA, as shown by sections stained with Spicer's Feulgen-methylene blue stain (Lillie 1965). In a qualitative study the cytoplasm of nurse cells had the highest concentration of RNA and the follicular cells the next highest, both being much higher than the oocyte. RNA seemed to be dispersed more homogeneously throughout the ooplasm in early stages than later. During yolk formation, it was retained in the background ooplasm, and could scarcely be demonstrated in the yolk spheres. The oocyte nucleus contained

RNA in a lobe-like structure. The nucleus of follicular cells contained its RNA in a single large, spherical structure. The RNA in the nurse-cell nucleus was not confined to a single structure, but rather, was localized in a ribbon-like chain of blobs.

Near the end of vitellogenesis the cytoplasm of follicular epithelium became more strongly stained , meaning that it contained more RNA. This coincided with the time of formation of vitelline membrane and chorion. At this time the RNA in the ooplasm was more centrally distributed, mainly around nucleus in perinuclear granules, than through the rest of the ooplasm (Fig. 22). The ooplasm remained stained even after egg maturation, although it was less intense. Whether the decreased staining reaction in the older oocyte was the result of an actual reduction in the amount of RNA present or because of dilution by other cytoplasmic materials has yet to be determined.

The Fat-body

When the female blackfly had just emerged, there was a large amount of larval fat-body present in the abdomen (Fig. 23). As the fly grew older, the larval fat-body gradually decreased in size while the eggs increased (Fig. 24). When the oocytes reached stage 13, there was still considerable larval fat body tissue present, particularly in the thoracic region. After the eggs had matured, about 5 days after emergence, there were only a few patches of larval fat-body remaining in the

thoracic region and around exocuticle of the female abdomen (Fig. 25). The larval fat-body almost disappeared from flies six days after emergence.

A comparison in the amount of larval fat-body remaining in females after certain time intervals related to the stages of egg development is summarized in Table II.

In larvae, pupae and the newly emerged adults as well as the adults which were several days older, the larval fat-body cells stained strongly with fast green (pH 1. 2), indicating an abundance of protein. They were positive to lipid stains of Sudan black B and oil red O. Although the larval fat-body decreased in size as the oocyte developed, the protein and lipids present decreased proportionally more rapidly during the same period. In larvae, pupae and newly emerged adults, the larval fat-body cells were faintly PAS-positive and this was unchanged by diastase digestion. When different stages were compared, the larvae were more strongly PAS-positive than the pupae, and the pupae more than the newly emerged flies. After the oocyte had developed to stage 11, two days after adult emergence, the fat-body cells were barely PAS-positive while the yolk spheres were strongly PAS-positive.

Table II

Amount of larval fat-body remaining in females fed sugar and water only, related to the stages of egg development, and time intervals after adult emergence

Stage	Number of days after adult emergence						
	0	1	2	3	4	5	6
9 - 10	++++(10)*						
9 - 11		++++(9) ++(1)					
10 - 12			+++(8) ++(4)				
11 - 13				+++(1) +(12)			
12 - 14					++(9) +(5)		
13 - 14						+(1) ±(5)	
14							±

Note: ++++ = much fat-body; ± = trace of fat-body; * Number of females showing each condition are in brackets

Fig. 1 Camera lucida drawing of a longitudinal section through an ovariole of a late larva of S. vittatum, showing a group of cells surrounded by a layer of follicular epithelium, the beginning of an egg chamber.

Fig. 2 Camera lucida drawings of the follicles of an early and a late pupa of S. vittatum

A. Follicle of an early pupa. The nucleus of the nurse cell and oocyte are similar.

B. Follicle of a late pupa. The nurse cell nuclei have multiple nucleoli; whereas the oocyte nucleus possesses a single nucleolus. The cell membranes of the nurse cells were unclear.

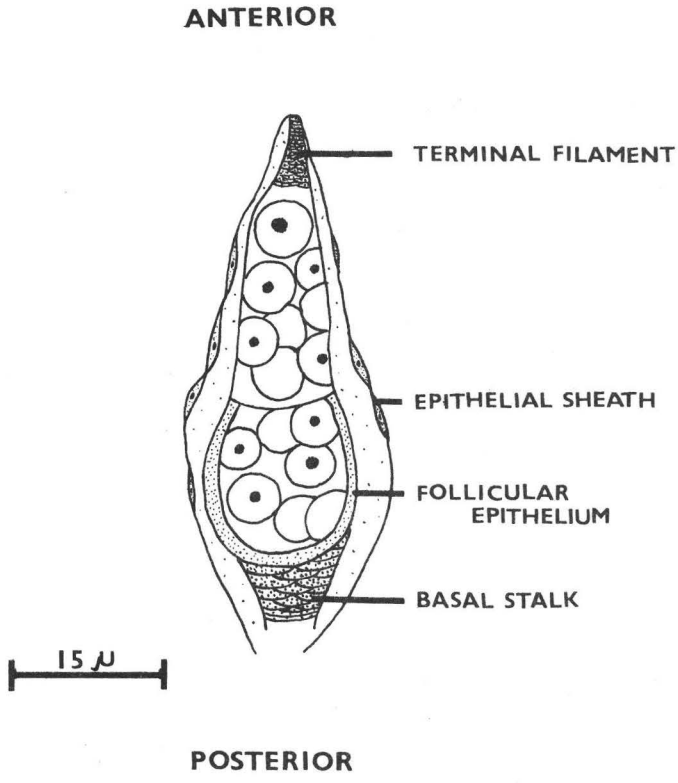

ANTERIOR
TERMINAL FILAMENT
EPITHELIAL SHEATH
FOLLICULAR EPITHELIUM
BASAL STALK
15 μ
POSTERIOR

FIG. 1

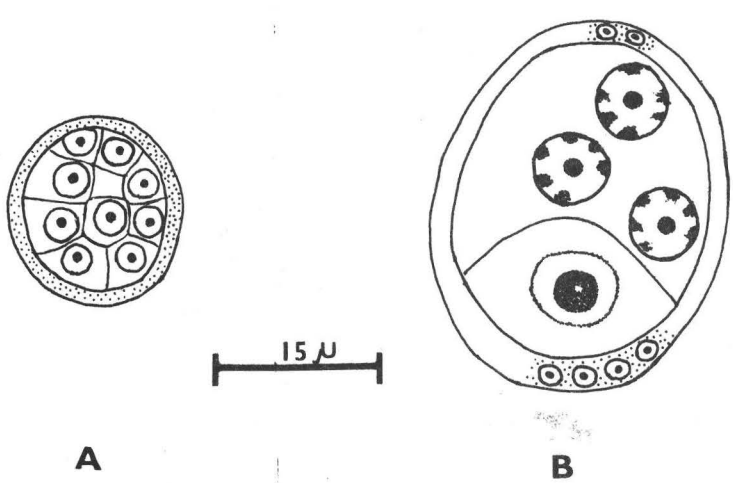

15 μ
A
B

FIG. 2

Fig. 3 Camera lucida drawing of a longitudinal section of an ovariole of a one-day old Simulium vittatum female from a hematoxylin-eosin preparation. Note the perinuclear granules in the oocyte.

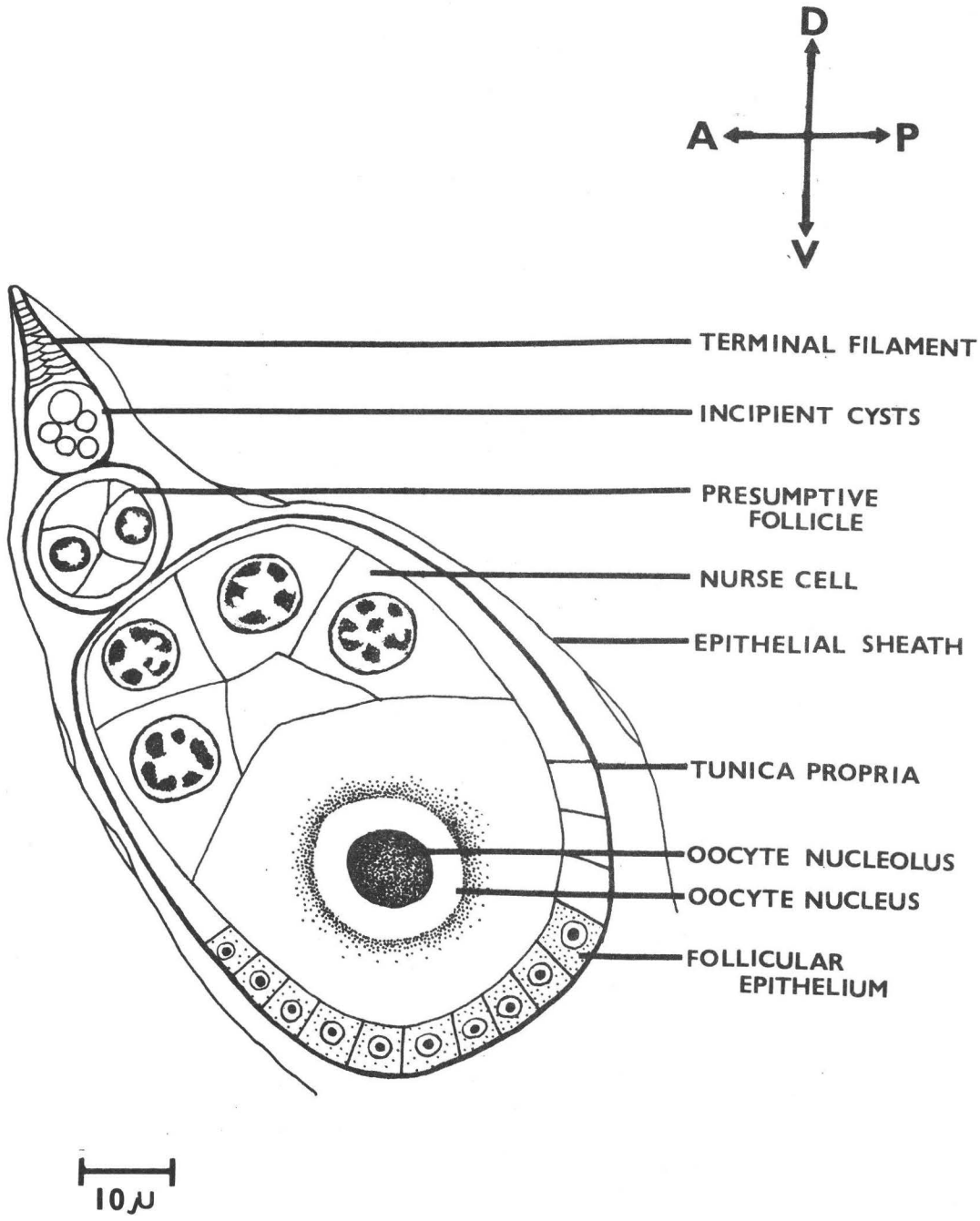

D
A
P
V
TERMINAL FILAMENT
INCIPIENT CYSTS
PRESUMPTIVE FOLLICLE
NURSE CELL
EPITHELIAL SHEATH
TUNICA PROPRIA
OOCYTE NUCLEOLUS
OOCYTE NUCLEUS
FOLLICULAR EPITHELIUM
10µ

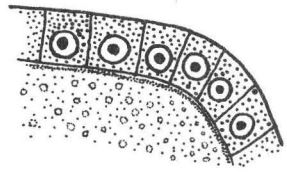
FIG. 3

Fig. 4 The follicular epithelium over the oocyte at three stages during vitellogenesis in S. vittatum

A. Columnar follicular epithelium at stage 10. Follicular cells are secreting droplets that become the vitteline membrane.

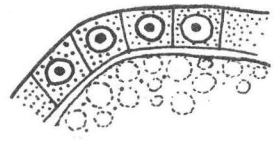
B. Cuboidal follicular epithelium at stage 11. Vitelline membrane complete around oocyte.

C. Flattened follicular epithelium at stage 12. Chorion complete around oocyte.



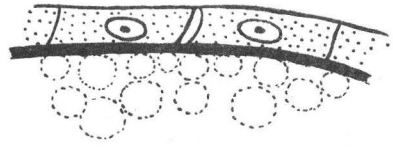
STAGE 10

A



STAGE 11

B



STAGE 12

15 μ

C

FIG. 4

Fig. 5. Diagrammatic representation of distribution of lipid granules (dark dots) in the oocyte of S. vittatum

A. Before carbohydrate-protein yolk formation. Oocyte is the largest cell.

B. After carbohydrate-protein yolk formation. Note the yolk spheres and enlarged oocyte nucleus.

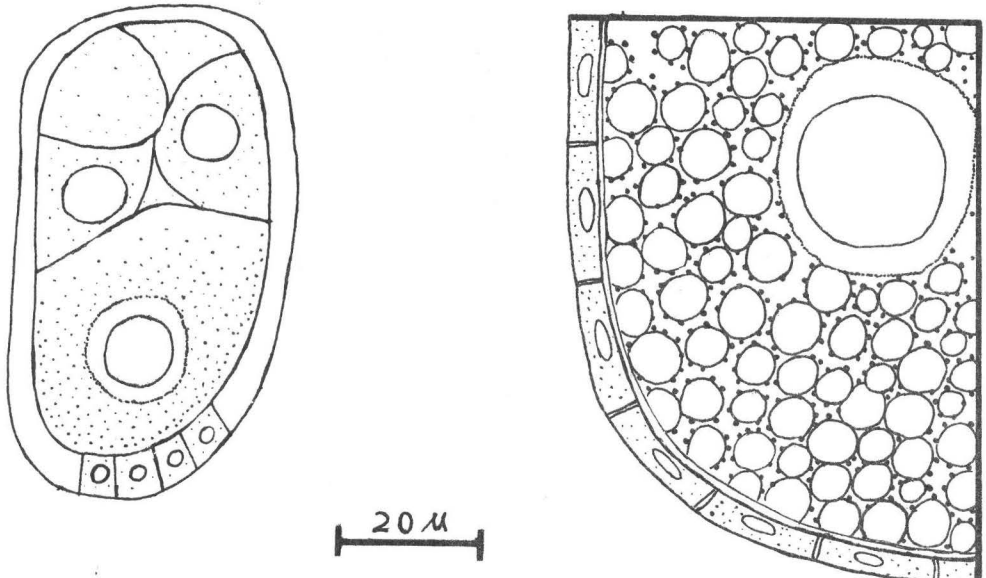


FIG. 5

Fig. 6. Volume of the follicle at different stages of development.

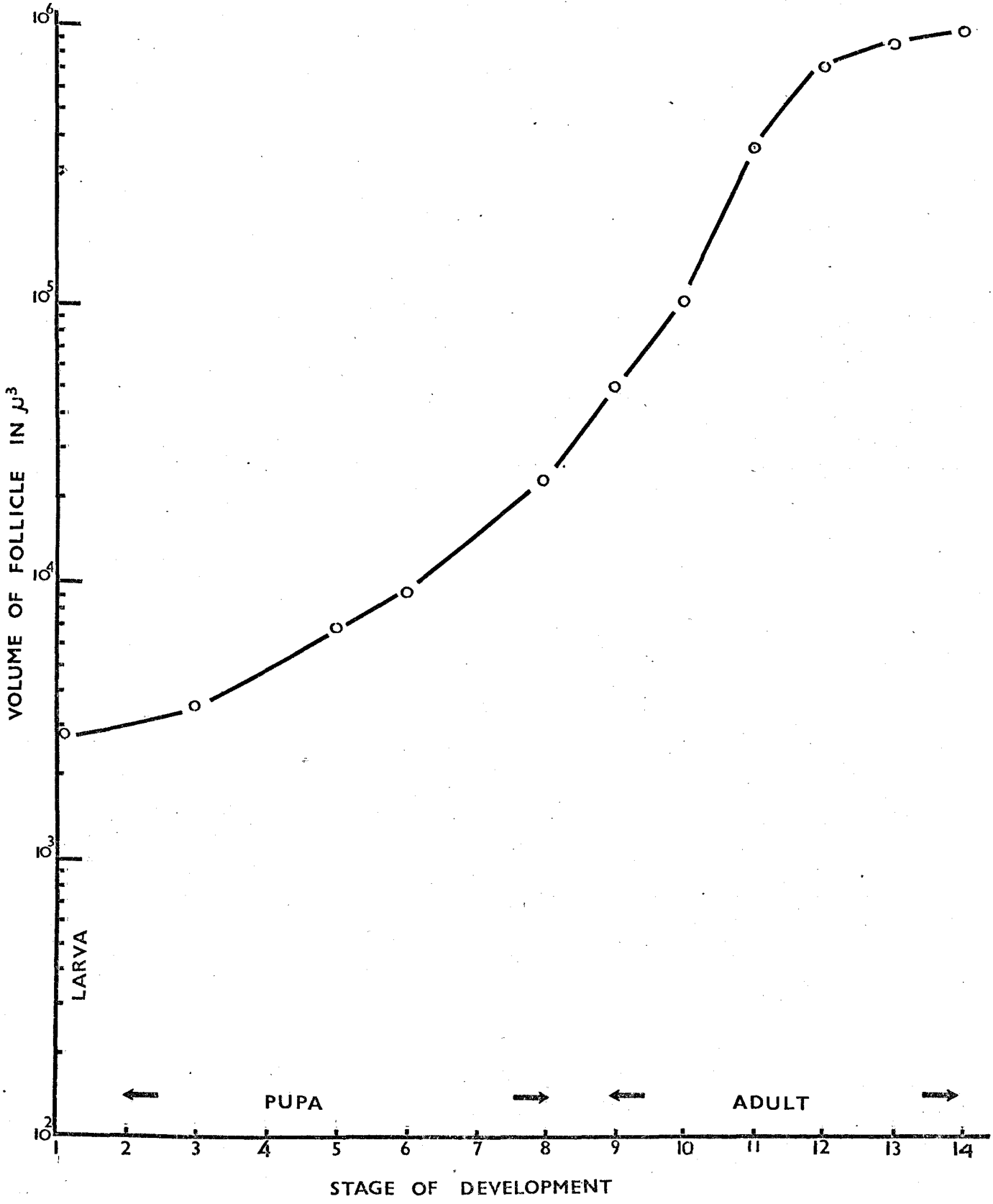

VOLUME OF FOLLICLE IN μ^3
10^6
10^5
10^4
10^3
10^2
LARVA
PUPA
ADULT
1
2
3
4
5
6
7
8
9
10
11
12
13
14
STAGE OF DEVELOPMENT

Fig. 7 Volume of oocyte and its nucleus as related to stage of development of the follicle

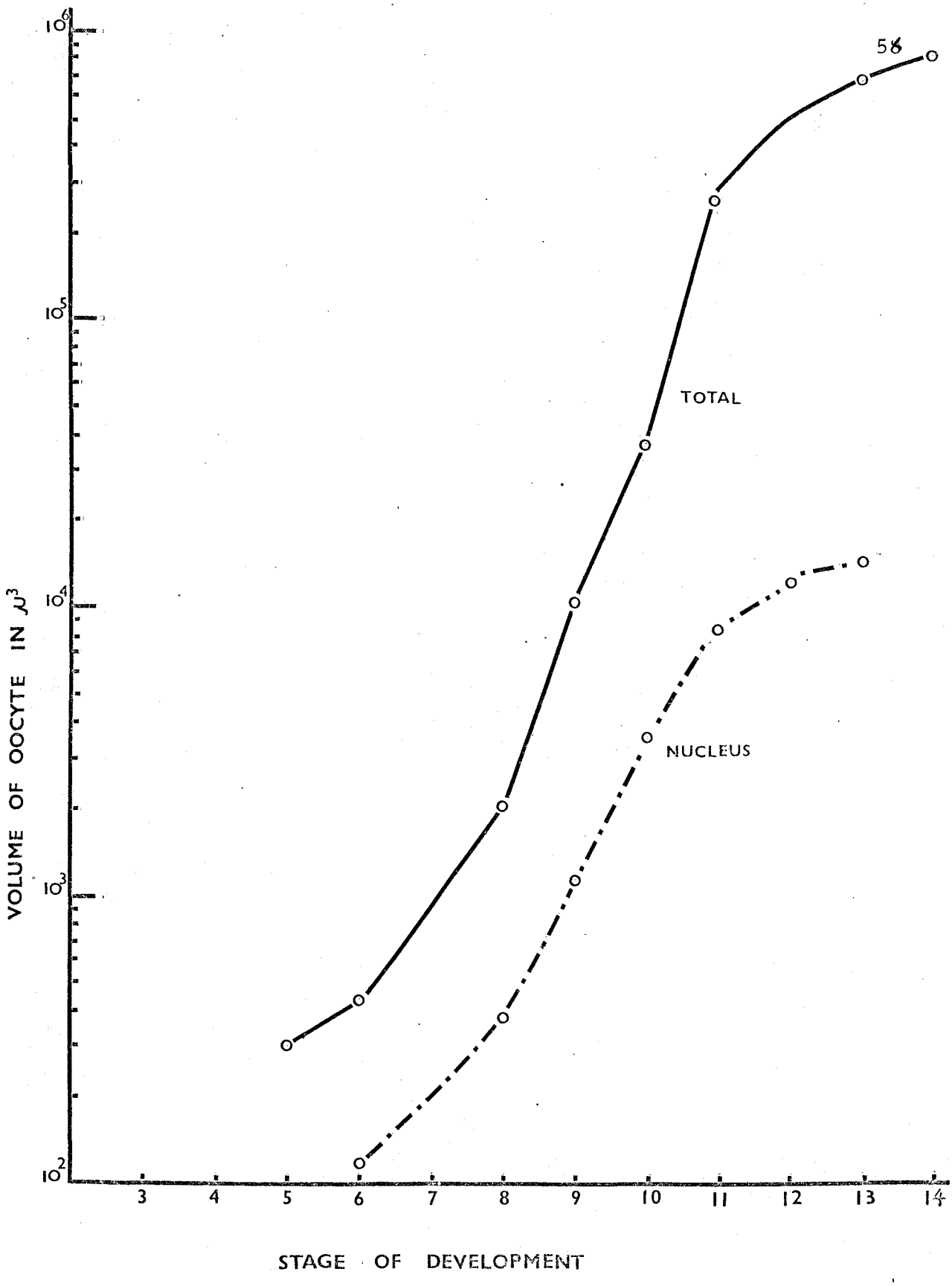

VOLUME OF OOCYTE IN μ^3
10^6
10^5
10^4
10^3
10^2
TOTAL
NUCLEUS
3
4
5
6
7
8
9
10
11
12
13
14
STAGE OF DEVELOPMENT

Fig. 8 Maximum cross-sectional diameter of the oocyte at different stages of development

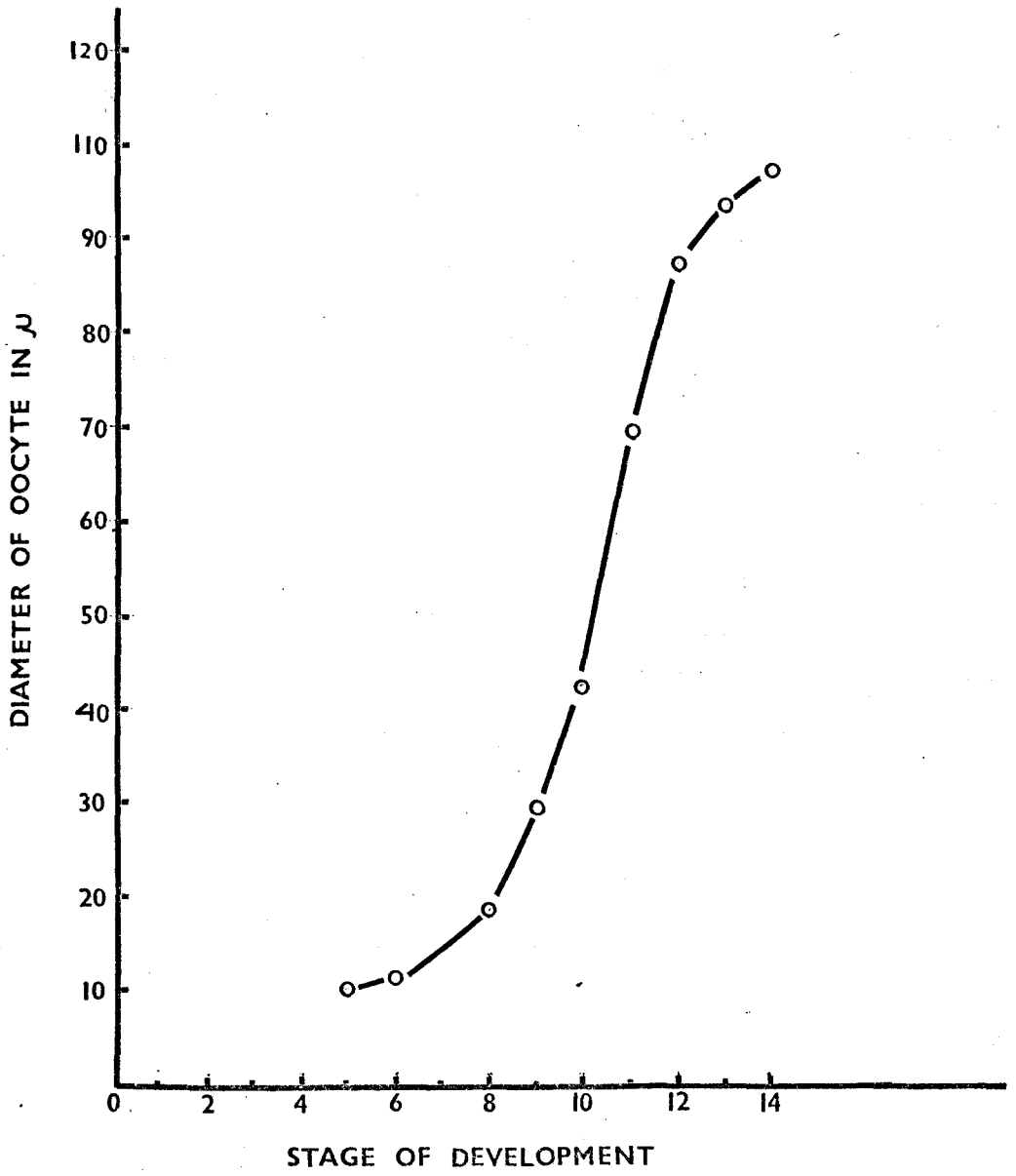

DIAMETER OF OOCYTE IN μ
120
110
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
STAGE OF DEVELOPMENT

Fig. 9 Volume of total 15 nurse cells and of their nuclei as related to the stage of development of the follicle

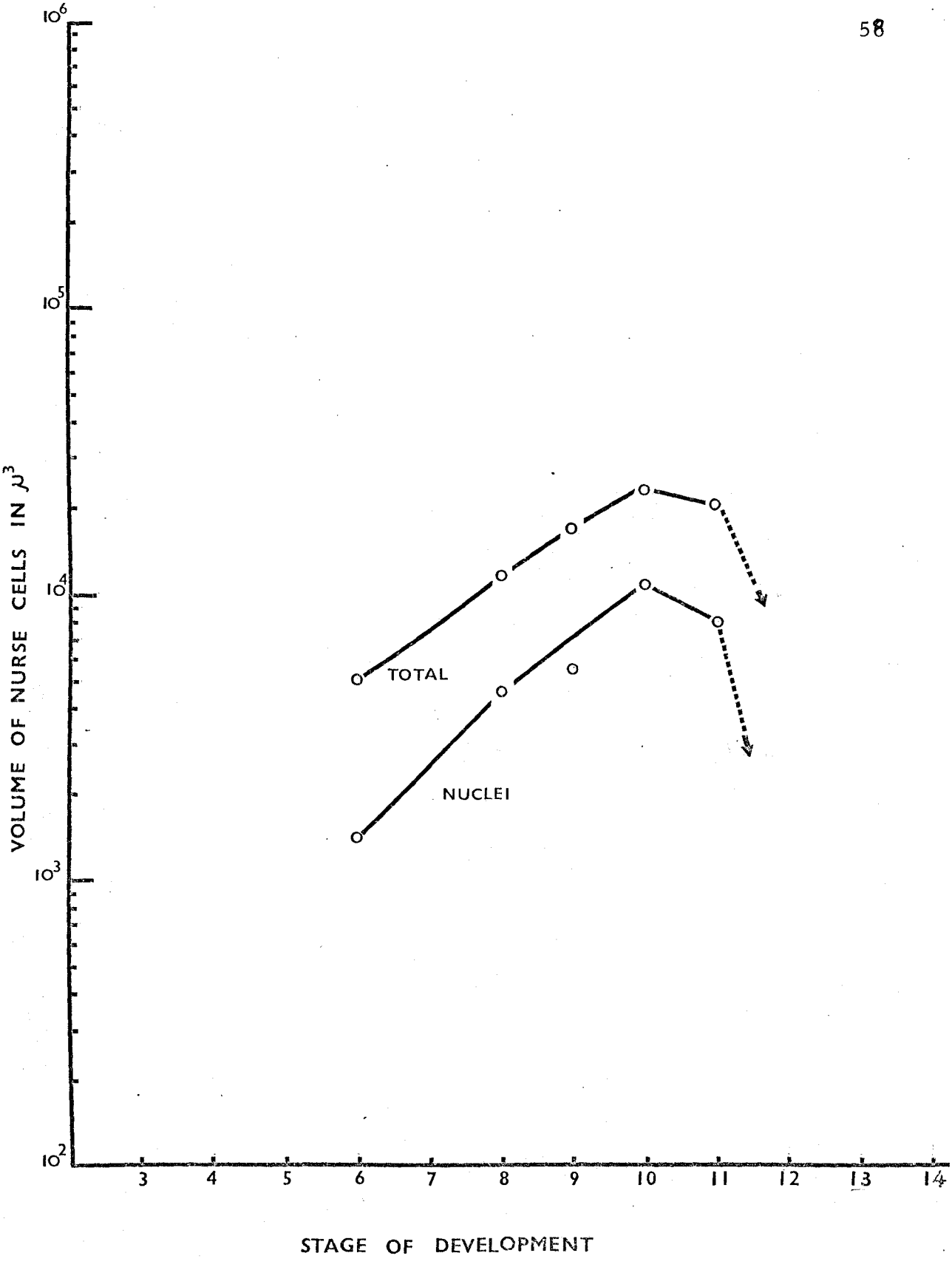

10^6
10^5
10^4
10^3
10^2
VOLUME OF NURSE CELLS IN μ^3
TOTAL
NUCLEI
3
4
5
6
7
8
9
10
11
12
13
14
STAGE OF DEVELOPMENT

Fig. 10 Height of the follicular epithelium surrounding the oocyte and the nurse cells at different stages of development

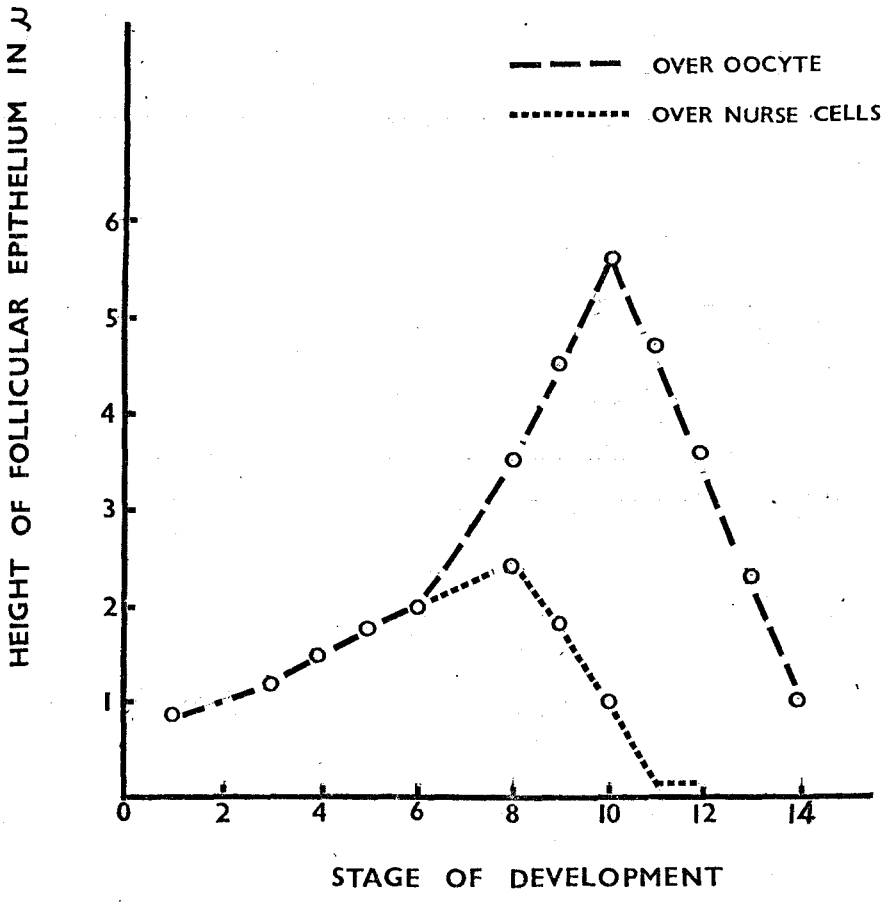

HEIGHT OF FOLLICULAR EPITHELIUM IN μ
OVER OOCYTE
OVER NURSE CELLS
1
2
3
4
5
6
0
2
4
6
8
10
12
14
STAGE OF DEVELOPMENT

Fig. 11 Time after emergence at which the various oogenic stages were reached in females of S. vittatum maintained at 13-15°C.

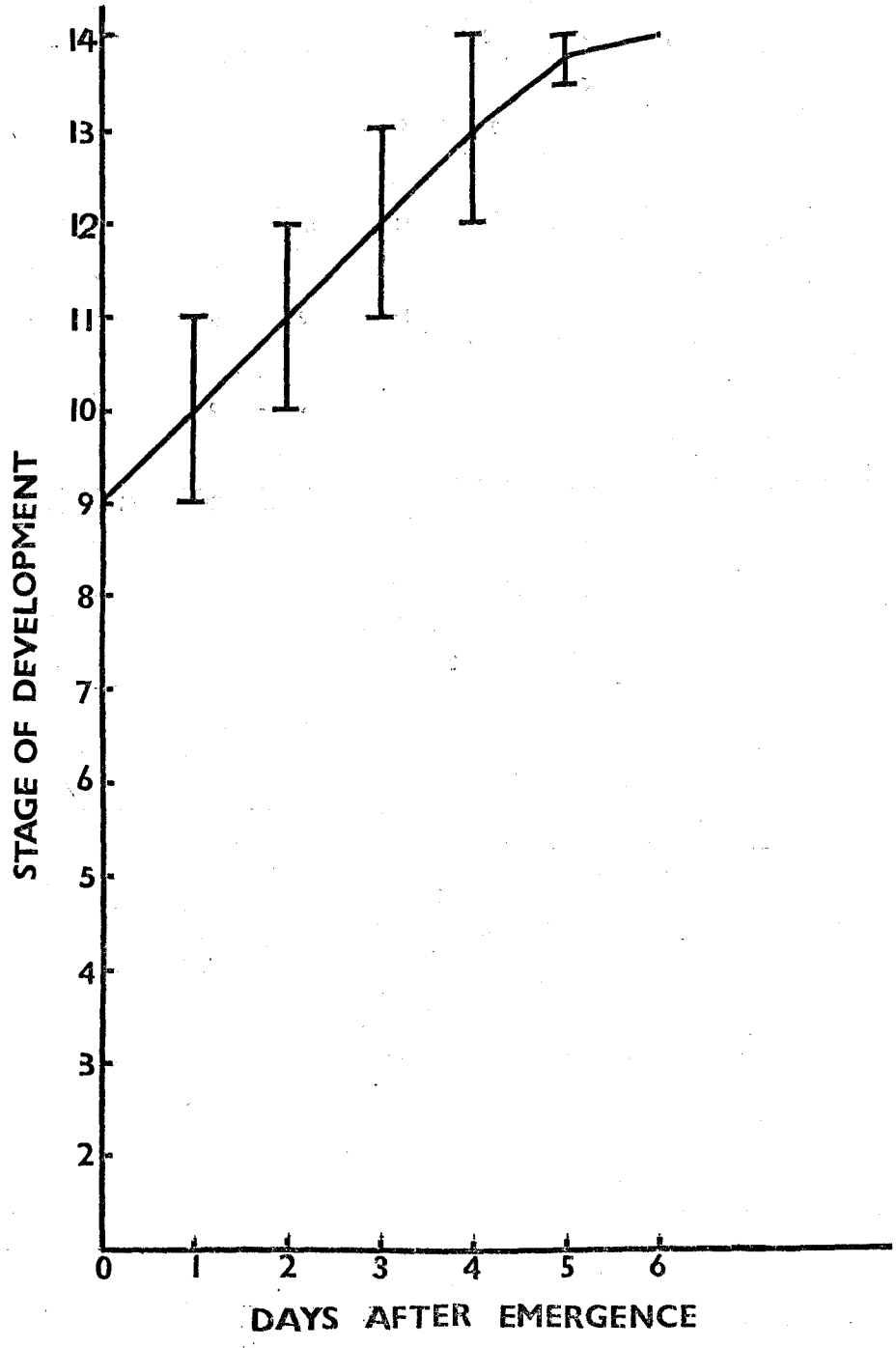
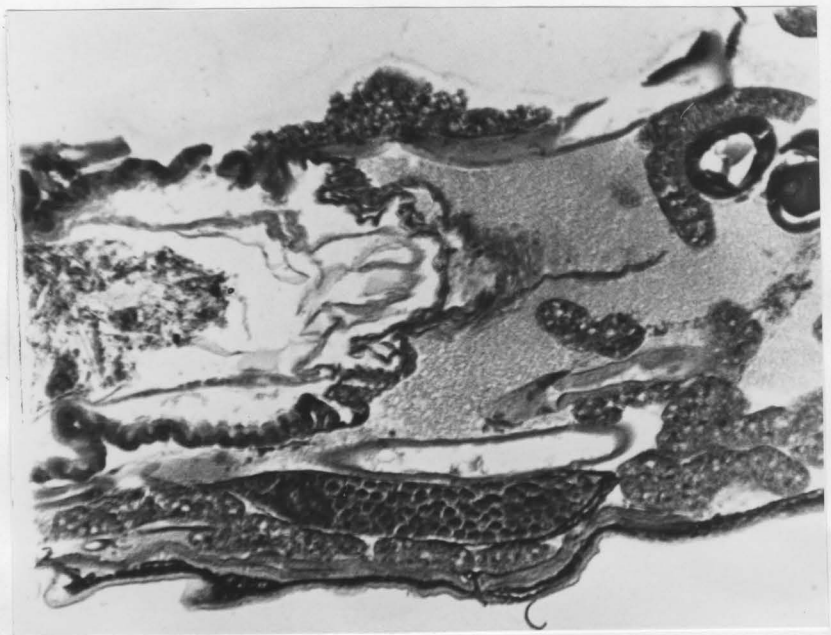

STAGE OF DEVELOPMENT
14
13
12
11
10
9
8
7
6
5
4
3
2
0
1
2
3
4
5
6
DAYS AFTER EMERGENCE

Fig. 12 Longitudinal section of the abdomen of a late larva of an S. vittatum female. The sausage-shaped ovary, lying ventro-lateral to the intestine, tapers anteriorly into a filament. H.E. 10μ. 450X

Fig. 13 Longitudinal section of the ovarioles of an early pupa, showing the spherical follicles at stage 5 separated from the germarium by the inter-follicular stalk. H.E. 10μ. 3880X



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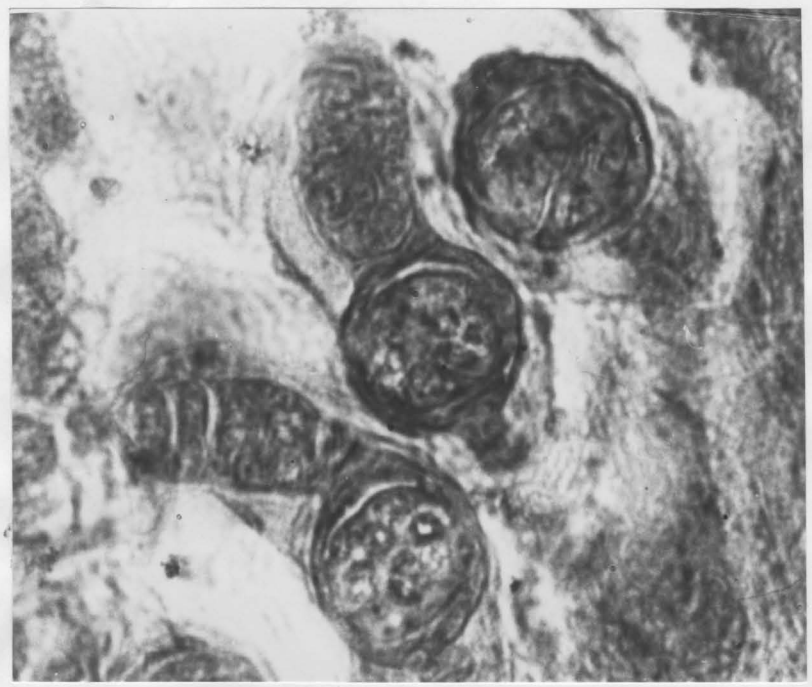
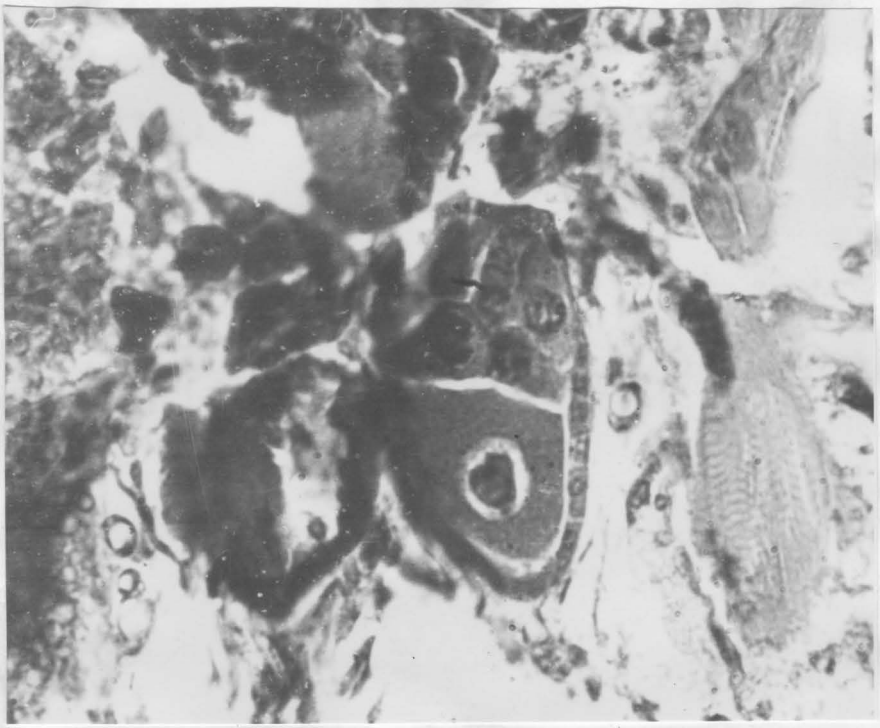


13

Fig. 14 Longitudinal section of an ellipsoidal follicle of a newly emerged adult, its oocyte makes up half of the total volume of the egg chamber. H. E. 10μ. 1720X

Fig. 15 Longitudinal section of a follicle of a newly emerged adult at stage 9, around the oocyte nucleus is a thin layer of perinuclear granules which stain blue with hematoxylin-eosin. H. E. 10μ, 1600X



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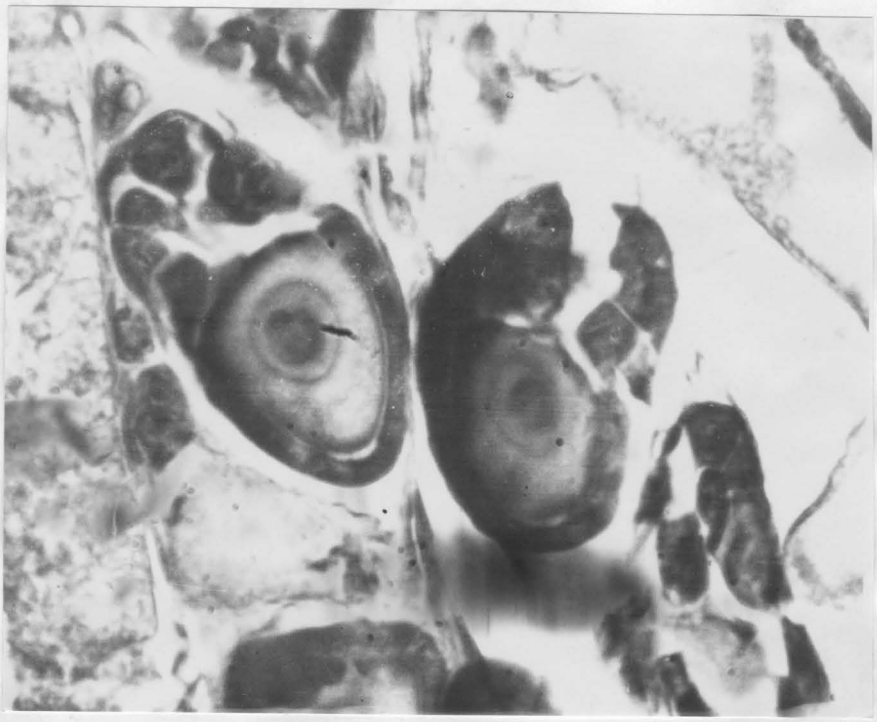
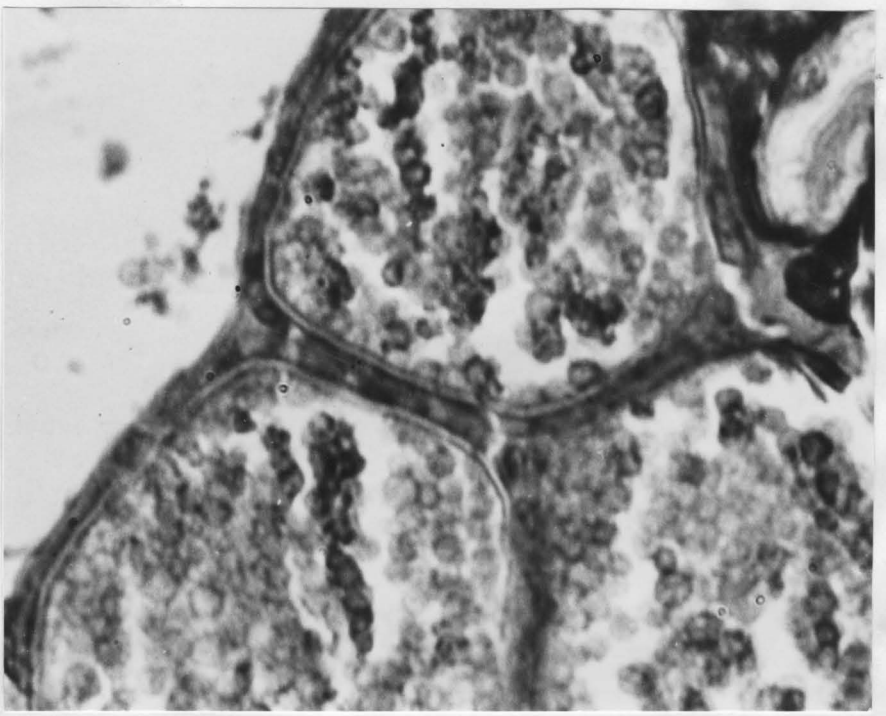


15

Fig. 16 Cross section of an oocyte at the end of sta 12. The completed chorion lies between the vitelline membrane and the flattened follicular epithelium. H. E. 10μ. 2580X

Fig. 17 Cross section of an oocyte at stage 12. Its nucleolus sometimes appears as a horse-shoe shaped structure. H. E. 10μ. 4250X



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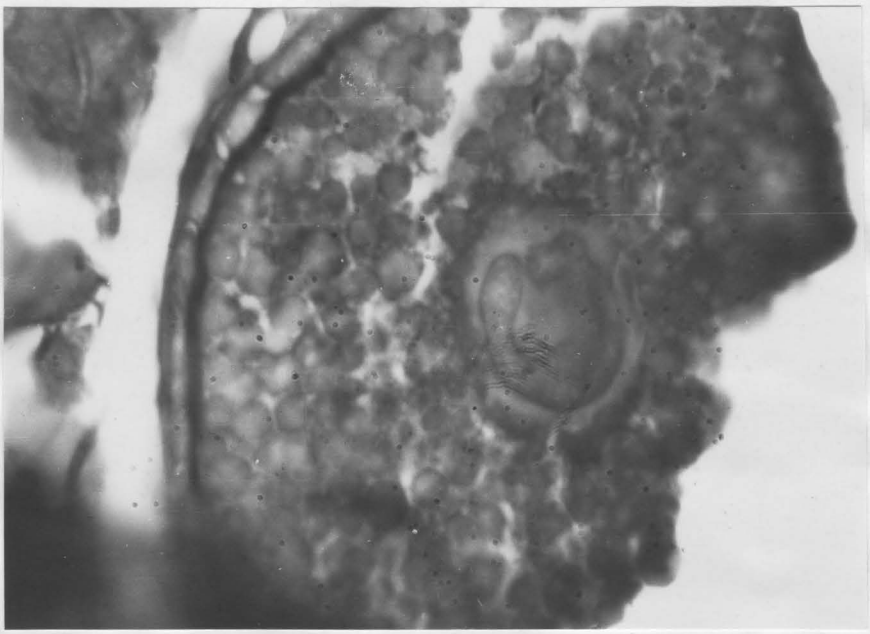
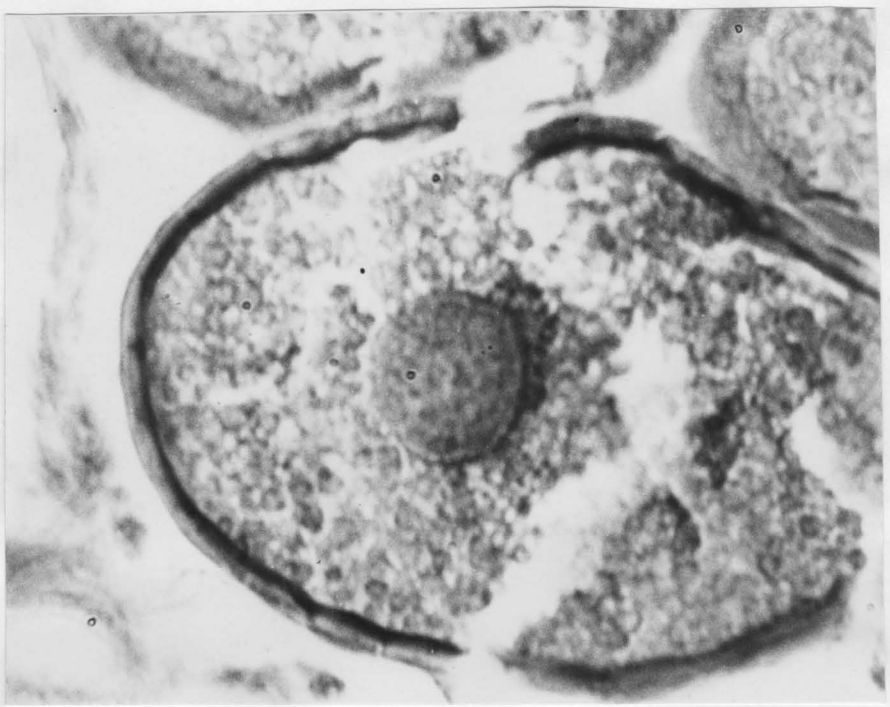


17

Fig. 18 Cross section of an oocyte at stage 12, showing its nucleolus having different stainability. H. E. 10μ. 2500X

Fig. 19 Cross section of a resorbing oocyte of a stage-12 ovariole. The follicular epithelium has thickened and vacuolated, the nuclei lose their precise arrangement. H. E. 2580X



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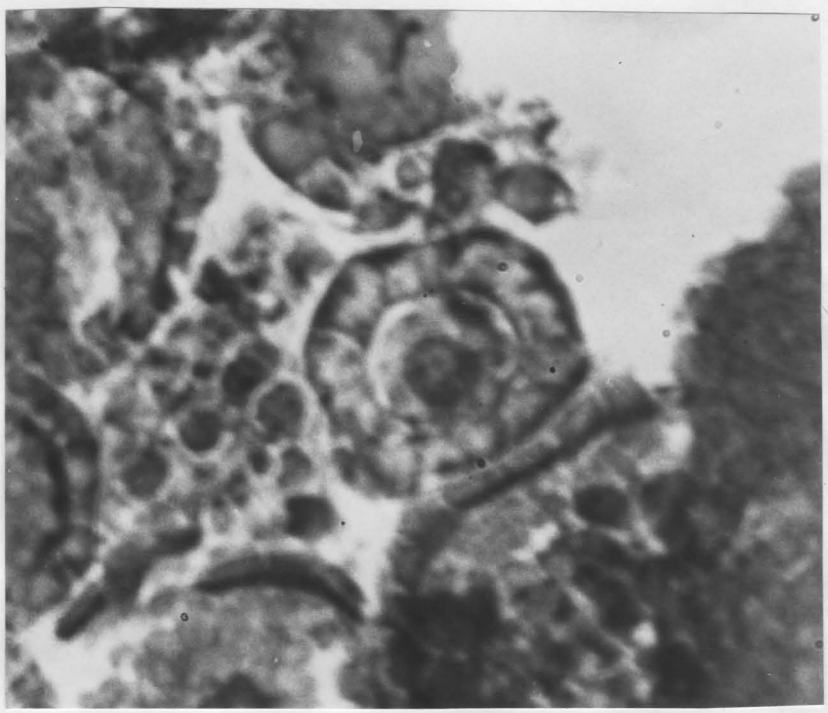
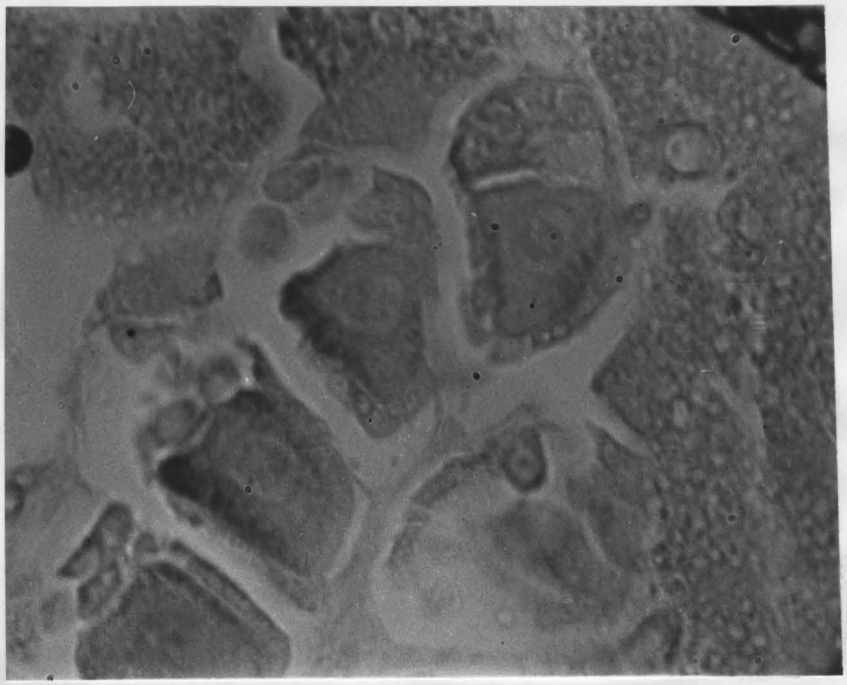


19

Fig. 20 Longitudinal section of a follicle at the end of stage 9, PAS-positive bodies first arise in the peripheral ooplasm adjacent to the follicular epithelium and later spread to the interior of the cell. PAS 8μ. 2000X

Fig. 21 Longitudinal section of a follicle at the end of stage 10, showing ooplasm completely filled with large PAS-positive yolk spheres. PAS 10μ, 2150X



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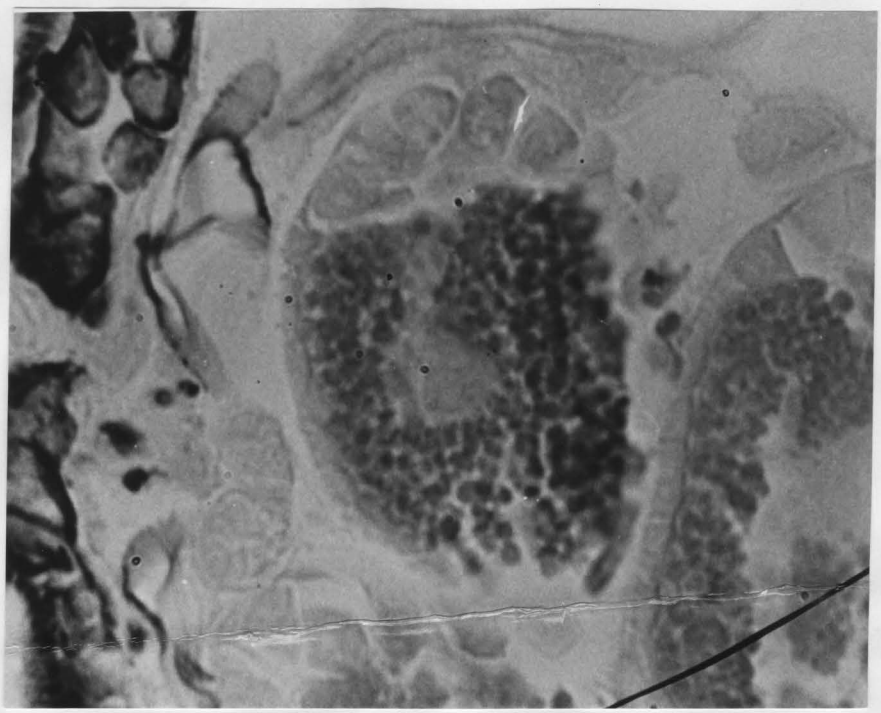
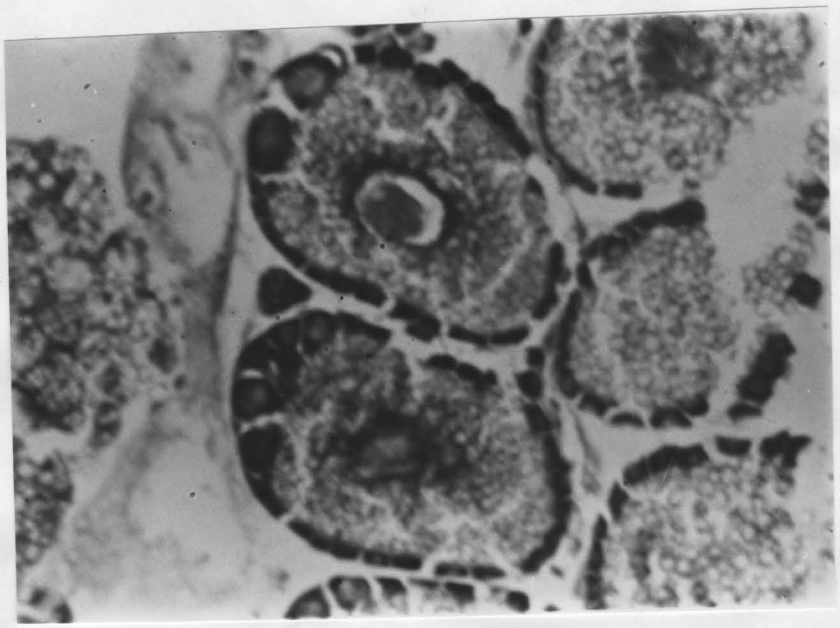


21

Fig. 22 Longitudinal section of a group of follicles of stage II, the perinuclear region of the ooplasm contains closely packed granules which appear more concentrated than in the other region of the ooplasm. Spicer's Feulgen methylene blue. 7u, 730X

Fig. 23 Longitudinal section of the abdomen of a newly emerged female black-fly, showing the large amount of larval fat-body with half-matured eggs. H. E. 10μ, 450X



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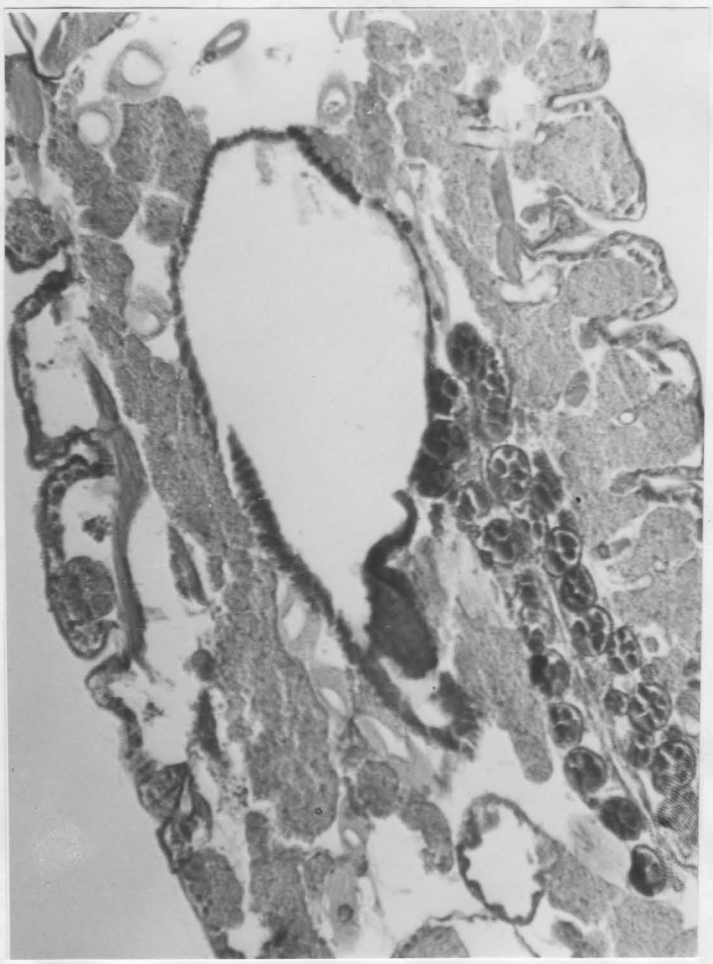
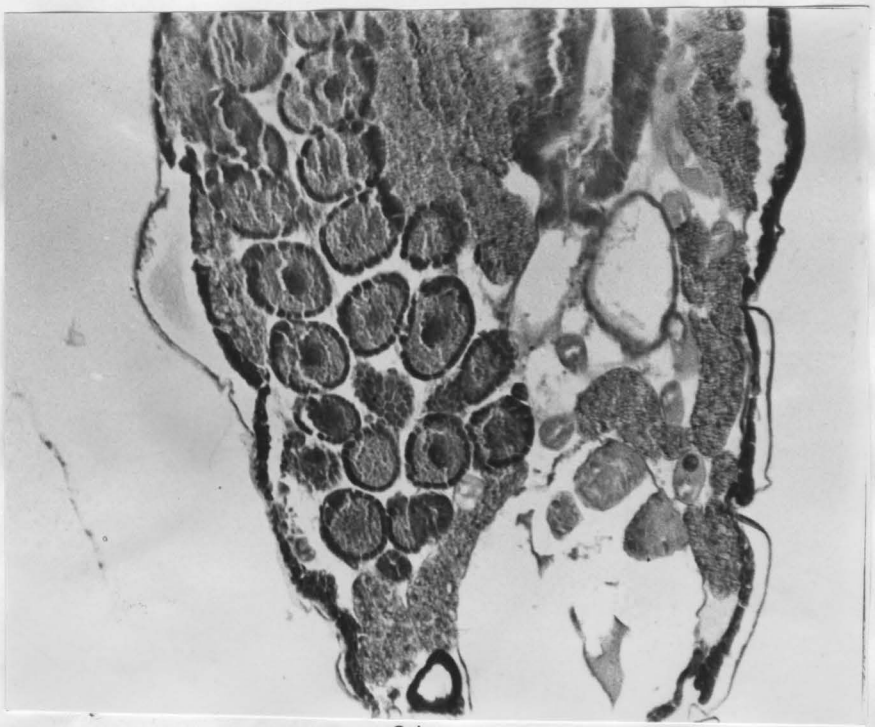


23

Fig. 24 Longitudinal section of the abdomen of a 2-day old female, fed sugar and water, with the follicle at stage 11, and less fat-body tissue than in Fig. 23. H. E. 10μ. 500X

Fig. 25 Longitudinal section of the abdomen and thorax of a 5-day old female with mature eggs and its larval fat-body almost disappeared. H. E. 10μ, 350X.



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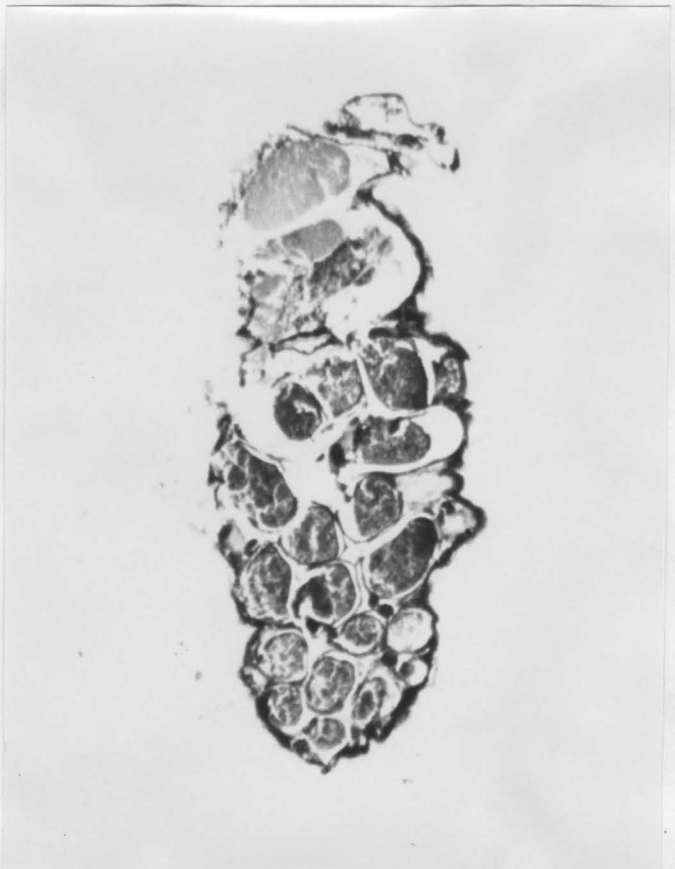

25

DISCUSSION

Introduction

Simulium vittatum was a suitable black-fly species in which to study oogenesis, as it occurred abundantly in the Hamilton region for most of the non-winter season and also it could be easily maintained from the late larval or early pupal stage until the end of the first gonadotrophic cycle in the adult (see Methods). Being autogenous, the adult females needed only sugar and water to facilitate the first cycle of oogenesis.

In general, the aspects of oogenesis in S. vittatum were similar to those of Musca domestica (Goodman et al. 1968) and Drosophila melanogaster (King et al. 1956, King and Devine 1958), although certain important differences occurred.

Timing of Oogenesis

In this black-fly, cytoblasts are first produced during the late larval period, whereas in Drosophila the same step is taken in the pupal period (King and Aggarwal 1965). During cystocytic division, the cells became smaller and smaller with each division until the 16-cell

stage was reached. According to Koch and King (1966), it is because a given daughter cell produced during cystocytic division does not grow to double its birth volume before it divides again. Before the larva pupated, the first egg chamber was already formed.

In the newly emerged female of the autogenous S. vittatum, the development of the follicle was already at stage 9. Certain other simuliid species have the eggs mature in emerging females (Davies and Peterson 1956 , Davies et al. 1962). By contrast, such females of the anautogenous S. venustum Say had follicles much less developed (eggs half the size of those of S. vittatum females) (Davies and Peterson 1956). In the anautogenous strain of the house-fly used by Goodman et al. (1968), the follicles did not develop beyond stage 7 or 8 until the female was fed amino acids or protein in addition to sugar and water.

It took 5-6 days for S. vittatum eggs to become mature at 13 - 15°C., which is shorter than the time required for blood-fed females of anautogenous simuliid species to mature their eggs (Davies and Peterson 1956). The oocytes of S. vittatum increased greatly in size during the adult period. It was repeatedly observed (from histological sections) that in the early stages the size of the eggs of the same batch of a given fly was similar and that all eggs matured simultaneously, which was also observed for the house-fly (Goodman et al. 1968). However, it was found that, when blackflies of the same age were compared, their eggs

were at different stages of development. This variance may be influenced by factors, such as nutrition, temperature, humidity and the degree of crowding during larval life.

In the later stages of egg development (stage 11-14), after the beginning of yolk formation, there were some flies which showed a few of their follicles being resorbed by the follicular epithelium. This is similar to the house-fly, where, when considering the first ovarian cycle, it usually occurred in late stages of development (stage 10-14) (Goodman 1963; Goodman et al. 1968). However, in the house-fly resorbtion of eggs was more frequent in later cycles. Resorbtion may occur because of nutritional factors and possibly in the blackfly, although the female may have received adequate nutrition in the larval stage to bring an oocyte to the point of yolk deposition, this oocyte might be unable to compete with other oocytes developing in the same fly.

Cytological Changes during Oogenesis

There were about 150 ovarioles per ovary as based on the number of eggs in gravid females of S. vittatum (Davies and Peterson 1956). Each black-fly ovariole contained two egg chambers developing at one time, whereas in the house-fly there was a maximum of three (Goodman et al. 1968) and in Drosophila seven to eight (King et al. 1956), each at a different stage of development. As in Drosophila and Musca

domestica, yolk deposition occurred only in the most posterior egg chamber, and the development of the follicular epithelium was almost the same.

After the black-fly emerged, the follicular cells around the oocyte were tending to become columnar whereas those surrounding the nurse cells were beginning to flatten from cuboidal with less cells per unit area. This was because the follicular epithelium had accommodated itself to the increased volume of the nurse cells by a change in shape rather than by cell division and growth. As yolk formation commenced those follicular cells above nurse cells became exceedingly thin, while those above the oocyte became columnar. Brown and King (1964) presumed that nutrients in fluids bathing the egg chamber pass through this follicular epithelium into the cytoplasm of the nurse cells. Thus a thin follicular epithelium would favor the rapid uptake of precursors from the haemolymph (Goodman et al. 1968). At stage 10, the columnar follicular cells had reached their maximum height. The columnar cells function in the formation of the vitelline membrane and subsequently the chorion. The follicular epithelium over the oocyte became cuboidal again in late stage 10, continued to decrease in height in later stages, and became squamous during stages 13 and 14. This was probably again caused by stretching of the follicular epithelium so that it could accommodate itself to the tremendous surface area of

the oocyte by a change in shape rather than by cell division and growth.

The black-fly follicle was similar to that of the fruit-fly (King et al. 1956) and the house-fly (Goodman et al. 1968) in having the nurse cells adjacent to the oocyte and those in contact with the follicular epithelium the largest with the largest nuclei. Brown and King (1964) suggested that in a given nurse cell the volume of the nucleus was presumably proportional to the degree of endopolyploidy, and that it increased in proportion to the rate at which extraovariolar nutrients entered the cell and to the rate at which products, synthesised by the cell, were removed. Thus the greater the area of the cell surface which is in contact with the squamous follicular cells (through which the extraovariolar nutrient must pass), the faster the nurse cell will receive precursors; and also the closer the nurse cell is to the oocyte, the faster its synthetic products will be removed. Jacob and Sirlin (1959) reported that the nurse cell with the smallest amount of DNA is in the middle group of cells. This is because its plasma membrane is in contact with the smallest area of squamous epithelium and this cell receives less extraovariolar nutrients than the other cells. The rapid growth of the nurse cells, simultaneously with that of the oocytes, is indicative of their importance in oogenesis. In the black-fly, as in the house-fly (Goodman et al. 1968), the nurse cells reached maximum

size during the time of yolk formation and later they degenerated. Their active role in vitellogenesis was substantiated by their large size and that of their presumably polytene nuclei. Since retarded DNA synthesis is accompanied by a lowered rate of yolk formation, it suggests that vitellogenesis can only proceed to completion with the normal, elevated DNA concentration (King and Sang 1959).

After stage 10, the nurse cells stopped growing and began to degenerate. According to Goodman et al. (1968), the degenerating nurse cells of the house-fly greatly contributed to the growth in bulk of the oocyte during the late period of yolk formation and their contents presumably streamed through the cellular connectives to the oocyte (Bier 1963). These might also partly explain why, in the black-fly after stage 11, the nurse cells and follicular epithelium decreased greatly in size, and although the chorion was forming, the oocyte still continued to grow, but at a much reduced rate. The nurse cell contents reaching the oocyte might be utilized to produce proteins during the later stages of oogenesis and early embryogenesis. It is claimed that in the cricket, Gryllus domestica, nurse-cell nuclei enter the oocyte simultaneously with their cytoplasm and dissolve in the ooplasm (Durand 1955). King et al. (1956) indicated that, if indeed this does occur, it is not a general phenomenon since in Drosophila a residuum of the nurse cells, including their nuclei, remains outside the chorion at the conclusion

of yolk formation. This was also found in the house-fly(Goodman et al 1968), and the black-fly showed the same phenomenon.

After the developing oocyte has its total complement of yolk to supply for the embryo, it is not yet complete, it must be provided with a protective coating. This coating should permit the exchange of gases necessary for the respiration of the embryo and it also should protect the embryo from desiccation. It was considered that in black-flies the secretion of the vitelline membrane and the chorion was an important function of the follicular epithelium. Development of the vitelline membrane of the black-fly was similar to that in Drosophila (King and Koch 1963) and Musca domestica (Goodman et al. 1968). The chorion was laid down inside the follicular epithelium after the vitelline membrane was completed. The chorion of the black-fly was everywhere solid, differing from house-fly whose chorion had a prominent middle region (Goodman et al. 1968). According to Hinton (1969) the air spaces in the chorion of terrestrial eggs, such as those of the house-fly, are lacking in the eggs of flies that oviposit into the water, e.g. Chironomidae. Their eggs are embedded in a jelly-like substance, the jelly loses water slowly so that if they are subjected to some desiccation and the drying period is not too long, the egg is protected from desiccation.

Vitellogenesis

It is generally believed that in some insects protein yolk spheres in the eggs are formed from extraovarian proteins, which are taken up at the oocyte surface by micropinocytosis. For a number of insects the fat-body was demonstrated to be an important extraovarian site of yolk protein synthesis (Telfer 1965). Especially when comparing the autogenous S. vittatum with the anautogenous S. venustum, the former with bigger eggs and much more stored nutrient on emergence than the latter (Davies and Peterson 1956), it is reasonable to presume that the fat-body had an influence on yolk protein formation. The way in which the yolk accumulated in the oocyte of certain insects has been reviewed by Telfer (1965) and King and Aggarwal (1965). But it is still a question whether all the proteins of the yolk spheres come from an extraovarian site or not (Kessel and Beams 1963; King and Aggarwal 1965; Goodman et al. 1968). In S. vittatum, according to the structure of the follicular epithelium and the intimate association of the fat-body with the oocyte, we may assume that, at least during the growth of the oocyte, there is a transfer of materials through this follicular layer to the nurse cells and the oocyte for the supply of the basic substances which are converted into the egg's own needs. At the present time it is hard to say without a more detailed study whether these materials reach the ooplasm by pinocytosis or by other means of active transport during vitellogenesis.

In the black-fly, unlike Drosophila and Musca domestica, each oocyte nucleus contains a large nucleolus and there is a special perinuclear zone of densely packed granules in the cytoplasm. This perinuclear plasm was especially rich in RNA. According to Kessel (1968), this perinuclear region in jellyfish oocytes appeared, under the electron microscope, to have ribosomes more concentrated here than in the more distal areas of the ooplasm. In the present study, it is uncertain whether some or all of the perinuclear granules are ribosomes, but it seems likely. In the black-fly, during late yolk formation, this perinuclear plasm became more prominent. These special granular accumulations in the boundary zone of nucleus and cytoplasm of oocyte may result from the migration of materials from the nucleus to the cytoplasm as in the coelenterate medusa (Kessel 1968). It is possible that in the black-fly there was some interaction between the nucleus and cytoplasm of oocyte, and that during the late phase of yolk formation the necessary intra-ooplasmic synthetic machinery, for at least a portion of its yolk components, was contained within the oocyte.

Histochemistry of Oogenesis

Because the yolk spheres of the black-fly oocyte were positive to PAS and these PAS positive bodies were diastase-resistant and stained with fast green (pH 1.2), it may be that the carbohydrate

is bound with the protein yolk globules to form a carbohydrate-protein complex. That the yolk spheres of S. vittatum contain a carbohydrate-protein complex is similar to the reports of other insects (Bonhag 1955, 1959; King 1960; Seshachar and Bagga 1963). These authors concluded from a combination of staining reactions that these yolk spheres contained either a glycoprotein or a mucoprotein. Although Seshachar and Bagga (1963) did find some minute glycogen granules applied to the larger yolk spheres of the dragonfly, these were not found in the mature egg. These findings differ from the recent reports of King and Aggarwal (1965) and Goodman et al. (1968). They found glycogen deposits in the oocyte of a saturniid moth and the house-fly, which persisted until the end of oogenesis. In addition to the diastase-sensitive granules, Ramamurty (1968) found that the scorpion-fly contained another diastase-resistant fraction which was the carbohydrate-protein complex.

The absence of the PAS-positive material from the nurse cells suggests that they have little to do with the carbohydrate nourishment of the oocyte as has been reported for the simplest type of polytrophic ovary in the earwig, Anisolabis maritima (Géné) (Bonhag 1956) and the more complicated type in house-fly (Goodman et al. 1969).

No PAS-positive granules were observed in the follicular epithelium of the black-fly egg chamber that could possibly be regarded as the yolk precursors. However, the PAS-positive yolk bodies

always appeared first in the peripheral ooplasm from where they migrated into the central ooplasm. The appearance of PAS-positive yolk precursors initially at the periphery of the cell suggests the possibility of some contribution to the developing oocyte from the follicular epithelium. However, till now there is no cytochemical evidence of direct transfer of PAS-positive material from the follicular epithelium to the oocyte cytoplasm. It is possible that the blood sugar is transported through the follicular epithelium to the periphery of the oocyte, where it could be polymerized into carbohydrate molecules and then bound with protein as also described earlier in Periplaneta (Aggarwal 1960), a saturniid moth (Telfer 1961) and Callosobruchus (Aggarwal 1967). In these insects, the follicular epithelium acts merely as a transferring system as in dragonfly (Seshachar and Bagga 1963).

In the black-fly, S. vittatum, lipids were confined to relatively small spheres in the ooplasm, which was reported also for the mosquito, Culex, (Nath et al. 1958b), the house-fly (Goodman et al. 1968) and the roach, Periplaneta, (Nath et al. 1958a). In the polytrophic ovary of Culex, Nath et al. (1958b) found no lipid contributions being made by the nurse cells but instead that the follicular epithelium serves as the source of raw materials for lipid synthesis in the oocyte. However, according to Hsu (1953), the lipoidal precursors in the Drosophila oocyte were emitted by the nucleoli of the nurse cell nuclei. In the house-fly,

Goodman et al. (1968) found that lipoidal granules were present in the nurse-cell cytoplasm and were in transit into the oocyte. Since the nurse cells and follicular cells of S. vittatum contained lipoidal granules, it is possible that one or both of these cell types contributed to the formation of the lipoidal granules of the oocyte.

Bonhag (1955) emphasizes that there are two conspicuous localizations of proteins in Oncopeltus oocytes: one in the yolk spheres and the other in the cytoplasmic matrix. However, only in the latter is the protein always associated with RNA. Subsequent evidence has shown that these are proteins with two entirely independent origins. The formation of yolk spheres from extracellular proteins has already been surveyed (see Introduction). While one cannot exclude the possibility that ooplasmic proteins also are deposited in the yolk spheres, the absence of significant amounts of endoplasmic reticulum and Golgi material in many oocytes makes it unlikely that they make a major contribution. The only animal in which the oocyte itself is known to make a substantial contribution to the protein yolk spheres is the crayfish.

Since nucleic acids are constantly associated with the synthesis of protein, observations on RNA in the developing oocyte and its associated tissues are relevant to the discussion of the protein bodies. In S. vittatum histochemical tests reveal a demonstrable amount of

RNA in cells of all types in the follicle at all stages but the amount varied, as based on the relative intensity of staining. The nurse-cell cytoplasm was always Feulgen methylene blue positive, indicating much RNA and usually active synthesis. As it was also strongly fast green positive, the RNA in nurse cells seemed associated with the synthesis of protein. In the early oocytes, the ooplasm showed a relatively intense RNA although, as the oocyte grew, this showed a general decrease. It is possible that the high concentration of RNA in the cytoplasm of the early growing oocyte reflected an increase in the amount of RNA related to protein synthesis. The later decline in the amount of ooplasmic RNA was due to its dilution, because the size of oocyte increased greatly as it developed (Bonhag 1956). By the end of stage 10, the follicular epithelium became columnar and functioned in the formation of the vitelline membrane and subsequently the chorion, a function which coincided with the high concentration of RNA in cytoplasm of follicular cells. This agreed with the finding of King (1960) for the fruit-fly that the concentration of RNA is greater in the cytoplasm of the columnar follicular epithelial cells than in the nurse cell cytoplasm or ooplasm at the same time. From the results of autoradiographic studies in the house-fly and fruit-fly Bier (1962, 1963) and King (1959) suggested that, as in many biological systems, the high content of RNA in the follicular epithelial cells is

concerned with the protein synthesis. Whether this is a sufficient explanation of the intensive RNA and protein synthesis occurring in these cells is still a matter of conjecture. There are some insects with polytrophic ovaries that contain RNA in their yolk spheres. This finding has been reported for earwigs, Labidura, (Nath, Gupta and Aggarwal 1959), for the silk moth, Bombyx mori, (Aggarwal 1962), and for the saturniid moth, Hyalophora cecropia (King and Aggarwal 1965). King and Aggarwal (1965) suggested that these yolk spheres are organelles which can synthesize polymeric molecules. Whether this occurs in the yolk spheres of the black-fly oocyte needs further investigation.

Fat-body and Autogeny

It is generally believed that the fat-body undergoes changes associated with egg development. However, previous authors have mainly stressed the importance of nutrition and hormones in insect ovarian development and neglected to investigate the relationship between the fat-body and ovarian growth. Recently it has been pointed out that the fat-body plays an important role in oogenesis in certain insects. This was found in Simulium vittatum (Davies and Peterson 1956), Hyalophora cecropia (Telfer 1963; King and Aggarwal 1965), and Musca domestica (Goodman 1963).

Since the autogenous black-fly matures its first batch of blood meal, it is obvious that the developing eggs must utilize stored nutrients elaborated from materials ingested during the larval period. This is similar to Lepidoptera, because most adult Lepidoptera are unable to assimilate protein, and therefore the proteinaceous elements of the eggs are mostly drawn from the larval reserves. The fat-body seems to be the obvious depot for these reserves. As eggs develop in the Lepidoptera, the size of the fat-body diminishes. The newly emerged female of Musca domestica (with oocyte at stage 4) and also those of S. vittatum (oocyte at stage 9) have large amounts of larval fat-body. Most of the house-fly larval fat-body disappeared two days after emergence and the ovaries hardly developed on the sugar water diet (Goodman 1963), whereas in S. vittatum with mature eggs (5 days after emergence), there were still a few patches of fat-body tissue remaining even without a blood meal. Therefore the house-fly must be supplied with other amino-acid nutrient in order to mature their eggs. Indeed we may presume that the difference in the amount and type of the reserves and the duration of time which reserves are retained during oogenesis might be one of the reasons for the occurrence of autogenous species. This may explain the reason why S. vittatum starts its egg development early from late larval period, and the abdomen of the newly emerged fly has oocytes at an advanced stage (stage 9) and a large amount of larval fat-body. It

may be that, if the oocyte fails to reach this advanced stage, the stored nutrient in the fat-body is probably insufficient to provide precursors for adult females to mature their eggs. Therefore, presumably the reserves in the fat-body can be transported from the fat-body cells into the blood, passed from the haemolymph through the membrane investing the oocyte, and into the ooplasm, and can form yolk bodies there capable of being hydrolyzed during subsequent embryogenesis. Such a sequence has been demonstrated in other insects (Coles 1964, King and Aggarwal 1965).

In this study it was shown that the larval fat-body in the late larva, the pupa and the adult female black-fly contained mostly protein, since it was strongly fast-green positive and lipids but probably no glycogen. Although the fat-body showed only faintly PAS-positive, this condition remains after diastase digestion. It may be that the black-fly larval fat-body contains a little muco- or glycoprotein. The amount of these materials decreased from larva to adult. Although some of these materials must have been used for maintenance, most of them were probably utilized for maturing eggs. Since no free glycogen appeared in the larval fat-body, it may explain why, soon after emergence, the black-fly had to feed on carbohydrate, as an energy source, similar to house-fly (Goodman 1963). If this was not supplied, the fly soon died.

Concluding Remarks

Much work has been done on the Diptera oogenesis, but only certain basic patterns of maturation and yolk deposition are known up to now and no complete process of change has been determined as yet. Since the ooplasm is the main nutritional substrate upon which the embryo must draw during its development, information on the origin, distribution and nature of yolk is of importance to studies of embryonic nutrition. Although the nurse and follicular cells play some part in the transport and synthesis of yolk, the contribution is complicated by time. Also from this investigation it seems that in the nematocerous black-fly, unlike certain cyclorrhaphous flies, the oocyte functions in synthesizing some protein itself, at least for a time during oogenesis. A more detailed study is needed, especially concerning the precise structural and chemical changes in the ooplasm which assures the initiation and completion of oogenesis. Certainly still much remains to be learned, before the scattered bits of information can be formed into a cohesive explanation of the site and mechanism of yolk formation, even for the Diptera.

SUMMARY

1. Each ovary of Simulium vittatum consisted of about 150 polytrophic ovarioles. The cytoblasts were first produced during the last larval period, and oocytes matured about 5-6 days following adult emergence.

2. The oocytes increased greatly in size during the adult period, mainly due to the accumulation of yolk. Simultaneously with the increase of the whole oocyte, its nucleus and nucleolus enlarged.

3. The occasional resorption of oocytes occurred generally during the later stages of oogenesis (stage 11-14) and presumably resulted from nutritional competition.

4. In a given fly all the eggs ordinarily reached maturity at the same time. However, flies of the same age differed in the stage of follicle development, which was considered to be related to larval nutrition or other environmental factors.

5. The rate of disappearance of larval fat-body in the adult female was slow compared to the house-fly, and there were a few patches remaining even after egg maturation (5 days after fly emergence). This is doubtless related to autogeny in the first gonadotrophic cycle of S. vittatum.

6. Yolk bodies, containing a carbohydrate-protein complex, first appeared at the peripheral of ooplasm adjacent to the follicular epithelium by stage 10, but later they were larger and migrated centrally and the whole oocyte became PAS-positive. Free glycogen could not be detected in the oocytes at any stage during oogenesis.

7. The nucleus and cytoplasm of all cell types of the follicle contained protein. The concentration varied little during the different stages except that follicular epithelium contained a higher amount of protein during the formation of vitelline membrane and chorion. During vitellogenesis the protein of the oocyte occurred almost entirely in the yolk spheres.

8. Lipids were present as granules in the follicle at all stages of growth. The lipoidal granules were dispersed in the ooplasm before, during and after yolk formation. No lipid was detected in the yolk spheres of the oocyte.

9. RNA was present in the cytoplasm and nucleus at all stages of follicular growth and appeared in higher amount in the follicular cells during the formation of vitelline membrane and chorion.

10. Preliminary observations indicated that perinuclear granules of the oocyte contained RNA, which might relate to the protein synthesis by the oocyte.

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