A study on the postembryonic ovarian development and vitellogenesis of Aulocara elliotti (Thomas) (Orthoptera, Acrididae)
by Roger Allen Leopold

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Entomology
Montana State University

Abstract:
The morphogenesis and histogenesis of ovarian tissue of Aulocara elliotti (Thomas) were examined throughout the nymphal and prereproductive stages® Differentiation; of the rudimentary ovariole into two functional zones, the germanium and vitellarium, occurs during the second instar. In those oocytes which are to he oviposited first, there is a considerable amount of cytoplasmic augmentation while they reside in the nymphal vitellarium. During the fifth instar a cuboidal epithelium differentiates from prefollieular tissue surrounding oocytes at the proximal end of the ovarioles. In the adult, two layers of muscle tissue are formed between the outer connective tissue sheath and the epithelial lining of the oviducts before the first clutch of eggs are to be laid. Between 8 and 10 days are required after adult emergence to complete the first reproductive cycle. After vitellogenesis, follicle cells around the posterior end of each proximal oocyte enlarge considerably before secreting a specialized chorionic cap overlaying the site of the future hydropyle. Presence of residual pigmentation is one of the gross differentiating factors between a resorbing oocyte and a normal degenerating follicle following ovulation, A cytological and cytochemical study of adult ovarioles was made with special emphasis on the process of vitellogenesis. During oocyte maturation the presence of Feulgen-positive material within oocyte nuclei diminishes, A heavy concentration of RHA is present in the portion of cytoplasm of the follicle cells closest to oocytes experiencing yolk deposition or chorion formation, A major portion of the yolk material deposited within oocytes appears to be a complex of protein and carbohydrate substances. In addition to the protein-carbohydrate fraction, there are significant amounts of acid and neutral mucopolysaccharides within the yolk granules. Although the deutoplasm of the oocytes is highly PAS-positive, the cytoplasm of surrounding follicle cells is PAS-negative.

It could not be determined whether rate of vitellogenesis or oocyte resorption was a function of the amount of light received per day when 127 females were examined seven days After emergence. There does appear to be trend for ovipositing females to lay fewer eggs upon exposure to shorter daylengths. Oviposition was not observed when females were maintained under constant light or three hours of light per day.

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ROGER ALLEN LEOPOLD

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

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ABSTRACT

The morphogenesis and histogenesis of ovarian tissue of Aulocara elliotti (Thomas) were examined throughout the nymphal and prereproductive stages. Differentiation of the rudimentary ovariole into two functional zones, the germarium and vitellarium, occurs during the second instar. In those oocytes which are to be oviposited first, there is a considerable amount of cytoplasmic augmentation while they reside in the nymphal vitellarium. During the fifth instar a cuboidal epithelium differentiates from prefollicular tissue surrounding oocytes at the proximal end of the ovarioles. In the adult, two layers of muscle tissue are formed between the outer connective tissue sheath and the epithelial lining of the oviducts before the first clutch of eggs are to be laid. Between 8 and 10 days are required after adult emergence to complete the first reproductive cycle. After vitellogenesis, follicle cells around the posterior end of each proximal oocyte enlarge considerably before secreting a specialized chorionic cap overlaying the site of the future hydropyle. Presence of residual pigmentation is one of the gross differentiating factors between a resorbing oocyte and a normal degenerating follicle following ovulation.

A cytological and cytochemical study of adult ovarioles was made with special emphasis on the process of vitellogenesis. During oocyte maturation the presence of Feulgen-positive material within oocyte nuclei diminishes. A heavy concentration of RNA is present in the portion of cytoplasm of the follicle cells closest to oocytes experiencing yolk deposition or chorion formation. A major portion of the yolk material deposited within oocytes appears to be a complex of protein and carbohydrate substances. In addition to the protein-carbohydrate fraction, there are significant amounts of acid and neutral mucopolysaccharides within the yolk granules. Although the deutoplasm of the oocytes is highly PAS-positive, the cytoplasm of surrounding follicle cells is PAS-negative.

It could not be determined whether rate of vitellogenesis or oocyte resorption was a function of the amount of light received per day when 127 females were examined seven days after emergence. There does appear to be trend for ovipositing females to lay fewer eggs upon exposure to shorter daylengths. Oviposition was not observed when females were maintained under constant light or three hours of light per day.

INTRODUCTION

The economic importance of the rangeland grasshopper, Aulocara elliotti (Thos.), has been verified by Pfadt (1949) and Anderson (1964). This insect is widely distributed over rangelands of the Great Plains region and has been observed to fluctuate greatly in numbers in certain areas of Montana.

In the last decade extensive studies have been conducted concerning the postovulatory physiology of developing A. elliotti eggs (Roemhild, 1961, 1965 a and b; Van Horn, 1963, 1964, 1966 a and b; Svoboda, 1964; Bunde, 1965; Laine, 1966). Only limited information is available, however, on the mechanisms by which eggs of this species are formed prior to ovulation. In order that these developmental processes indigenous to preovulatory eggs may be better understood, the present investigation of postembryonic ovarian development and vitellogenesis was undertaken.

Although embryonic development of female acridid gonads has been described by several workers (e.g. Graber, 1891; Roonwal, 1937; Nelsen, 1931, 1934b), ovarian development following hatching has received little attention. Heymons (1895) was probably one of the first to study post-embryonic development in an Orthopteran. Nelsen (1934b) followed ovarian development throughout embryogenesis and the nymphal instars in the Acridid, Melanoplus differentialis (Thos.). Both investigators noted that newly hatched female nymphs had relatively undeveloped gonads and that considerable morphogenesis and histogenesis of ovarian tissue occurred during the nymphal stages.

Phipps (1949) studied the gross structure of maturing adult ovaries

in seven Acrididae. He noted that several atypical conditions were displayed by adult ovaries during maturation, one of which appeared to be oocyte resorption. Singh (1958) made a histological investigation of degenerating ovarian follicles of Schistocerca gregaria (Forsk.). A comparison of postovulatory follicles and resorbed follicles was made. It was concluded that typical postovulatory follicles upon degeneration contained no pigmentation, whereas an orange-red pigment was present in degenerating follicles where oocyte resorption had occurred. Further study of resorbing oocytes revealed that the residual orange-red pigment within resorbed follicles was B-carotene (Lusis, 1963). Lusis also discovered that follicle cells acted as lecitholytic cells during the early stages of oocyte resorption, first breaking down the deutoplasm before they themselves degenerate.

The origin and composition of yolk has been studied in numerous insect species. However, the conclusions derived from these studies are diverse and, in some cases, contradictory. Because of the advancements made in the field of histochemistry during recent years, these techniques are now widely used in the study of insect vitellogenesis. Although yolk deposition in acridid insects has received limited attention, vitellogenesis in many other insect species has been studied using histochemical methods.

In Perplaneta orientalis Linn., Gresson (1931) detected no Feulgen-positive material within oocyte nuclei either before or during vitellogenesis. This does not appear to be the usual condition displayed by oocytes in

many of the other insect species studied. Instead, the nuclei of oogonia and young oocytes generally exhibit a strong Feulgen-reaction during the early stages of oogenesis and then the reaction gradually diminishes before yolk deposition is completed (Bonhag, 1959; Radecka, 1962; Weglarska, 1962). Nuclei of follicle cells ordinarily show no variation in the high level of DNA present throughout the cytoplasmic growth and yolk deposition in the primary oocytes.

Nucleolar extrusions from the primary oocyte nucleaus of the Oriental roach and the American roach have been reported to give rise to proteid yolk in the peripheral ooplasm (Hogben, 1920; Nath and Mohan, 1929; Gresson, 1931).

Hegner (1914) found that mitochondria at the periphery of developing oocytes of Leptinotarsa decemlineata (Say) increased rapidly in size and number and then formed large yolk bodies. Ranade (1933) studied vitellogenesis in Periplaneta americana (Linn.) and concluded that protein was produced by mitochondria and not by nucleolar emissions.

Yet another origin of proteid yolk was assessed in oocytes of Nepa cinera Linn. (Steopoe, 1926). Golgi bodies were thought to be responsible for the production of proteid yolk bodies in this species. In an earlier study of the same species of bug, Spaul (1922) proposed a nucleolar origin for the yolk bodies.

Bonhag (1959) and Radecka (1962) maintain that nucleolar emissions only serve to transfer material from the nucleus to the cytoplasms of the oocyte and do not give rise directly to proteid yolk. This view appears

to be consistent with the more recent understanding of protein synthesis.

In studying oogenesis in Tachycines asynamorus Adel., Radecka (1962) suggested that the follicle epithelium may contribute portions of the deutoplasmic substances to developing oocytes.

Other workers maintain yolk protein is synthesized elsewhere in an insect's body and is taken into developing oocytes by the process of micropinocytosis (Kessel and Beams, 1963; Roth and Porter, 1964; Anderson, 1964; King and Aggarwal, 1964; Stay, 1965). Telfer (1961) and Ramamurty (1963) suggested that yolk protein enters oocytes during vitellogenesis via the intercellular spaces of the follicular epithelium. It was inferred that there was little participation of the follicle cells in yolk deposition.

Lipoid yolk has also been thought to originate from a variety of sources. Numerous investigators have concluded that Golgi bodies within the oocytes develop into lipoid yolk (Nath and Mohan, 1929; Nath and Metha, 1929; Gresson, 1931; Gupta, 1958a). Most of these authors agree that lipoid yolk first appears in Golgi vacuoles, which swell and change into fat droplets. The fat droplets may remain surrounded for some time by an osmiophilic sheath.

Hsu (1952) observed a direct transformation of mitochondria into fat droplets in oocytes of Drosophilia melanogaster Meigen. Fatty yolk globules were suggested to arise in the ooplasm without relation to any of the formed elements within the oocyte (Steopoe, 1929).

Bonhag (1955b) stated that sudanophilic lipids of both non-phospholipid and phospholipid types appeared to have been contributed to the oocytes of

Oncopeltus fasciatus (Dallas) by the follicular epithelium.

Several workers have suggested that carbohydrate compounds in yolk exist as a protein-carbohydrate complex. Bonhag (1955a) postulated that yolk material, which could not be extracted with a methanol-chloroform solution, consisted of a mucoprotein or a glycoprotein. A similar conclusion was reached by Lusis (1963) when he studied oocyte resorption in Schistocerca.

Yolk material reacting positively to the periodic-acid Schiff test in oocytes of Gerris remigis Say was conjectured to be a mucoprotein, glycoprotein, or a neutral mucopolysaccharide in complex with protein (Eschenberg, 1965).

Although glycogen is one of the main storage polysaccharides of insects, it was not found in developing oocytes of Oncopeltus, Tachycines, or Gerris (Bonhag, 1955a; Radecka, 1962; Eschenberg, 1965). Bonhag (1956), however, found glycogen to be present in the older oocytes of Anisolabis maritima (Géné).

Insect vitellogenesis and oviposition have been found to be sensitive to a variety of extrinsic factors. Many insect species have been discovered to react in some manner to a changing photoperiod since Marcovitch (1924) first demonstrated the importance of daylength upon the appearance of sexual forms in aphids. Reproduction is acknowledged to be one of the physiological processes responsive to changing daylengths in numerous insect species.

In the laboratory, Schistocerca gregaria has been observed to enter

a reproductive diapause upon a lengthening of the daylight periods, whereas Nomadacris septemfasciata Serv. experiences a cessation of ovarian development when the photoperiod becomes shorter (Norris, 1957, 1962, 1965). Middlekauff (1964) has shown that rate of oocyte maturation increases with decreasing daylengths in adult Melanoplus devastator Scud. females.

Oocytes in Dytiscus and Leptinotarsa were observed to form and resorb continually when these insects were maintained under short daylengths comparable to those during winter months (Joly, 1945; De Wilde, 1954).

The mechanism for phtoperiodic control of insect reproduction appears to act through the neuroendocrine system. De Wilde and De Boer (1961) have demonstrated that oogenesis in Leptinotarsa is controlled by way of the brain through the corpora allata. It has been further observed that protein haemolymph concentration rises in adult females before oocyte yolk deposition and is depleted during each reproductive cycle (Hill, 1962; Highnam, Lusis and Hill, 1963; Orr, 1964; Engelmann, 1965). Thomsen and Möller (1959) have indicated that the neurosecretory substance is directly involved in stimulation of protein synthesis.

Highnam et al., (1963) have shown that the fewer the number of ovarioles in Schistocerca the fewer the number of resorbed oocytes. These workers contend that developing oocytes are in competition with each other for available haemolymph protein. Developing oocytes which do not acquire adequate material from the haemolymph are ultimately resorbed.

Using the aforementioned investigations as a background, the present

study on postembryonic ovarian development and vitellogenesis was initiated.

MATERIALS AND METHODS

General Morphology

Grasshoppers used for the morphological study of postembryonic ovarian development, with the exception of first instar nymphs, were collected from wild populations during the summer of 1965. First instar females were obtained from laboratory hatched eggs which had been collected from wild population sites during the fall of 1964.

In order that females with known ages could be obtained for each instar, only females which had hatched or had moulted in the laboratory were used. Newly hatched or moulted grasshoppers were placed into cages containing individuals of the same instar. Vials filled with Western Wheatgrass (Agropyron smithii Rydb.), which is the predominant plant in their natural diet (Anderson, 1964; Pfadt, 1949) were provided daily. Females were taken from these groups every other day and fixed. In this manner the sequential order of development could be followed during the nymphal instars to the adult stage. This study did not include developmental observations on adult females which had completed over seven days of imaginal life as their ovaries were arbitrarily considered to be mature.

Because ovaries of A. elliotti nymphs are small and rather difficult to remove from the younger grasshoppers, a transverse cut was made through the area between the first and second thoracic segments and that portion of the body caudad to the cut was immediately immersed in fixative in toto. Ovaries from teneral through seven-day adults were obtained by vivisection in a 0.65% Ringer's solution. Vivisection was accomplished by pinning the adult female specimen to a wax surface, submerging it with Ringer's

solution, and making a dorsal-longitudinal incision from the epiproct to the cervicum. The exposed ovaries were excised from the grasshoppers by cutting the lateral oviducts and removing the attached fat body and tracheae with the aid of a jeweler's forceps, microdissecting scissors, and a dissecting microscope.

Boiun's, Carnoy's (3:1), Sinha's, and Ammerman's fixatives were used in this investigation. Sinhas's fixative gave the best results for study of the first through fifth instar nymphs. This fixative enhanced sectioning the portions of the body containing the ovaries due to the softening effect it had on the exoskeleton. When ovariectomies were performed and only the excised ovaries fixed, Carnoy's and Ammerman's fixatives yielded the best structural detail. Fixation time for Sinha's Carnoy's and Ammerman's fixatives was 24, 3 and 2 hours respectively.

The fixed material was dehydrated and double embedded according to a modified version of the procedure by Humanson (1962) using a solution of methyl benzoate and celloidin. Due to the high alcohol content of both Sinha's and Carnoy's fluids, dehydration was begun with 95% instead of 50% ethanol. Paraplast (manufactured by Biological Research Inc.) was substituted for paraffin. Infiltration with three changes of Paraplast was executed in a vacuum in an oven at 55-57° C for one hour. The tissue was then embedded in Paraplast and stored until it could be cut with a rotary microtome. Serial sections of all developmental stages were cut at 6 μ and mounted on slides for this study.

The following stains were used: Delafield's hematoxylin and eosin,

Heidenhain's iron hematoxylin, and Mallory's triple connective tissue stain. For study of all stages of ovarian development, Delafield's hematoxylin and eosin proved to be the most helpful as a general stain for all ovarian structures. Mallory's triple connective tissue stain was useful in the examination of the adult ovaries in which yolk was being deposited.

Supravital observations were made with the aid of a dissecting microscope upon adult ovaries exposed by vivisection. Additional observations were made on adult ovaries which had been dissected out and immersed in the 0.65% Ringer's solution.

Cytology and Cytochemistry

For cytological and cytochemical investigations, ovarian tissue was obtained by dissection of females reared in the laboratory or collected from wild populations. Observations were made on ovaries from adult grasshoppers ranging from teneral to senescent forms. The dissection technique was the same as that described previously for adult forms.

Immediately after being dissected, the ovarian tissue was fixed according to the requirements of the staining methods to be used. All material was sectioned on a rotary microtome at 6 μ except for the study concerning the distribution of lipids in the ovarioles. This tissue was cut at 15 μ with a freezing microtome.

For demonstration of nucleic acids the following methods were used: 1) methyl green and pyronin Y for DNA and RNA as outlined by Barka and Anderson (1963); 2) gallocyamin-chrome alum for DNA and RNA according to

Einarson (1951) with the modification of Beswick (1958); 3) Feulgen's reaction by Barka and Anderson (1963) using Lillie's preparation of Schiff's reagent (1951b). For this procedure the hydrolysis time was ten minutes using 1.0 N HCl at 60°C. Alternate sections were counterstained in a 0.05% alcoholic fast green stain after treatment with Schiff's reagent.

Carnoy's solution and cold acetone were used as fixatives for the methyl green-pyronin Y method. Two hours in Carnoy's solution resulted in some loss of RNA. This decrease in staining intensity was not observed when the ovaries were fixed in three changes of cold acetone for 12 hours. To prevent loss of RNA which may occur after prolonged storage in alcohol (Brachet, 1953), material for this technique was embedded as soon as possible following fixation. Carnoy's solution was used exclusively as the fixative for the gallocyanin-chrome alum method. Susa's solution was used as the fixative for the Feulgen reaction. Fixation time for Susa's solution was 24 hours for all methods in which it was used as the fixative.

Control sections for the study of RNA were digested in ribonuclease for four hours at 37°C. Ribonuclease from bovine pancreas which had been recrystallized five times was used at a concentration of 0.3 mg/ml in deionized, glass distilled water adjusted to pH 6.8 with 0.1 N HCl. Unhydrolyzed sections served as the control for the Feulgen reaction.

For study of the general distribution of proteins, ovarian tissue was fixed in Susa's solution and stained with mercuric bromphenol blue (Mazia, Brewer, Alfert, 1953). There was a considerable loss of dye from the sections when the suggested phosphate buffer and succeeding dehydration in

ethanol was used. Consequently, the refinement of Bonhag (1955a) was used in which the buffer is omitted and dehydration is in tertiary butyl alcohol.

The localization of lipids in general was accomplished by using the sudan black B technique of Chiffelle and Putt (1951). The tissue was fixed with buffered 10% formalin for three hours and embedded in gelatin according to Culling (1957). Infiltration of the tissue with the 20% gelatin was increased to 24 hours at 45°C instead of the suggested 12 hours at 37°C. This modification was necessary in order that acceptable sections might be obtained. No alteration in staining intensity or locality was noted when this variation in Culling's procedure was employed.

For demonstration of unsaturated lipids, the osmic acid technique of Mallory (1944) was used. Even though osmic acid is one of the oldest lipid "stains", Bahr (1954) has questioned the specificity of this method because reducing groups of proteins and carbohydrates may yield a positive reaction. However, Adams (1960) has shown that preliminary elimination or blocking of these reducing groups does not diminish osmiophilia but the saturation of lipid -CH=CH- bonds causes its complete extinction. The tissue for this study was handled prior to staining in the same manner as that used with the Sudan black B procedure. The stained sections for both techniques were mounted in glycerin jelly.

Three staining techniques were employed in an effort to demonstrate the presence of glycogen in the ovaries. These staining techniques were: 1) Best's carmine after Humanson (1962); 2) The Bauer-Feulgen reaction of Bauer (1933); and 3) the PAS reaction with the dimedone (5, 5-dimethyl-1,

3 cyclohexadione) blockade as control (Bulmer, 1959).

Absolute alcohol and formalin (9:1) were used as the fixative for the first two methods and Carnoy's solution was used for the third method. Fixation time for all three methods was three hours.

Control sections for Best's carmine and the Bauer-Feulgen reaction were digested in Lillie's (1949) solution of malt diastase for one hour prior to staining. Additional sections were digested in saliva for one hour before treatment according to Best's carmine method. The control sections for the PAS reaction were placed in 5% acetic acid saturated with dimedone for six hours at 60°C after oxidation in periodic acid.

The PAS technique used in the attempt to demonstrate presence of glycogen and other carbohydrates having a positive reaction to this test is as follows: 1) the Paraplast embedding material was removed from the sections with benzene; 2) after immersion in absolute ethanol, the sections were coated with a solution containing 0.5% celloidin and then progressively hydrated to distilled water; 3) the sections were oxidized in 0.75% aqueous periodic acid for ten minutes; 4) the sections were washed in running tap water and placed in Schiff's reagent for 30 minutes; 5) the sections were bleached in a solution consisting of 10 ml of 10% sodium metabisulfite, 10ml N HCl, and 200 ml of distilled water; 6) after a ten minute wash in running tap water the sections were dehydrated in ethanol, cleared and mounted. Ovaries preserved with Carnoy's and Susa's fixatives were used for all carbohydrate analyses employing the PAS technique. No differences in staining intensity or locality could be

ascertained when one fixative was substituted for the other. Lillie's (1951b) formula for Schiff's reagent was used throughout the analysis for reactive carbohydrates.

Several controls were used in the effort to characterize the carbohydrates found to react positively using the PAS technique. These controls were: 1) sections stained without previous oxidation in periodic acid; 2) sections acetylated for two hours in a 2:3 mixture of acetic anhydride pyridine succeeded by deacetylation for 24 hours in a 1:4 mixture of NH_4OH (28%) and ethanol; 3) sections treated with a solution of 0.1 mg/ml pepsin in 0.01 N HCl at 37°C for two hours; 4) sections treated with a solution of 0.3 mg/ml bovine hyaluronidase in 0.1 phosphate buffer, pH 5.9, at 37°C for five hours; and 5) sections placed in a 1:1 mixture of methanol and chloroform at 60°C for 24 hours (Bonhag, 1955a). The coating of the sections with celloidin was omitted when enzyme digestion was utilized. Because sections were lost from the slides when they were treated with the pepsin solution, the Paraplast embedding material was not removed from the sections until immediately before they were mounted (Bonhag, 1955a).

Because protein and carbohydrate yolk granules appear to have similar locations within the more mature oocytes, a modification of the technique of Himes and Moriber (1956) was used to investigate this situation more closely. This technique is designed to display DNA, polysaccharides, and protein when present. The method was modified by omitting the azure A-Schiff reagent in order that only the presence of protein and carbohydrate

could be demonstrated. Ovarian tissue preserved with Susa's fixative was used in this study.

The alcian blue staining method of Steedman (1950), revised by Mowry (1956), was used to demonstrate the presence of acid mucopolysaccharides in ovarian tissue preserved with Carnoy's fixative. A 0.1% aqueous solution of toluidine blue was used for the metachromasia analysis. The sections were stained in this solution for one hour. Control sections were digested in hyaluronidase using the same procedure as described previously for PAS reactive carbohydrates. Some of the sections were examined under water immediately after staining, whereas others were examined following the dehydration, clearing and mounting procedure.

To observe intra-vital staining of the ovarioles, one µl of a 0.5% solution of trypan blue in 0.2 M phosphate buffer, pH 6.9, was injected into the haemoceles of A. elliotti females reared in the laboratory. Grasshoppers from this group were sacrificed at 8, 16, 24 and 33 hour intervals so that ovaries could be dissected and fixed in Susa's solution. The fixed material was then embedded in Paraplast, sectioned, affixed to slides, immersed in toluene to remove the Paraplast, and mounted. No further staining was employed subsequent to examination of these sections.

Photomicrographs were taken of stained sections resulting from the previous studies with a Leitz 4 X 5 camera through a Zeiss microscope equipped with Planapochromat and Neofluar objectives.

Photoperiodism

To examine the possibility that photoperiod may have some control

over the rate of vitellogenesis, frequency of oocyte resorption and frequency of oviposition, grasshoppers were collected from a wild population near Decker, Montana, and reared in the laboratory under a regime of 13 different photoperiods. Twelve of the 13 photoperiods were based upon a 24 hour day. The thirteenth photoperiod was used as a control and grasshoppers reared under it had constant light.

A battery of 24 cages was constructed in such a manner so that each of the 12 pairs of cages would have a different daylength (Fig. 1). A sliding plywood shield was designed so that on hourly intervals it would cover one pair of cages. When this shield had covered each of the 12 pairs of cages, it would then proceed to uncover one pair of cages every hour on the hour until all had been uncovered. The time required for the shield to traverse the distance from one end of the battery of cages to the other and back to its starting position was 24 hours. The 12 photoperiods resulting from this arrangement differed from each other by two hour intervals and ranged from 23 hours of light to one hour of light.

The sliding shield was powered by a 1/8 horsepower, split-phase electric motor. A relay system consisting of two relays governed the direction of movement for the shield. This relay system was wired in such a manner that one relay was in series with the motor windings causing it to go forward and the other relay was in series with the motor windings causing it to go backward. A toggle switch in series to the relay system was tripped by the shield at either end of the battery of cages. This action energized the corresponding relay allowing the shield to reverse

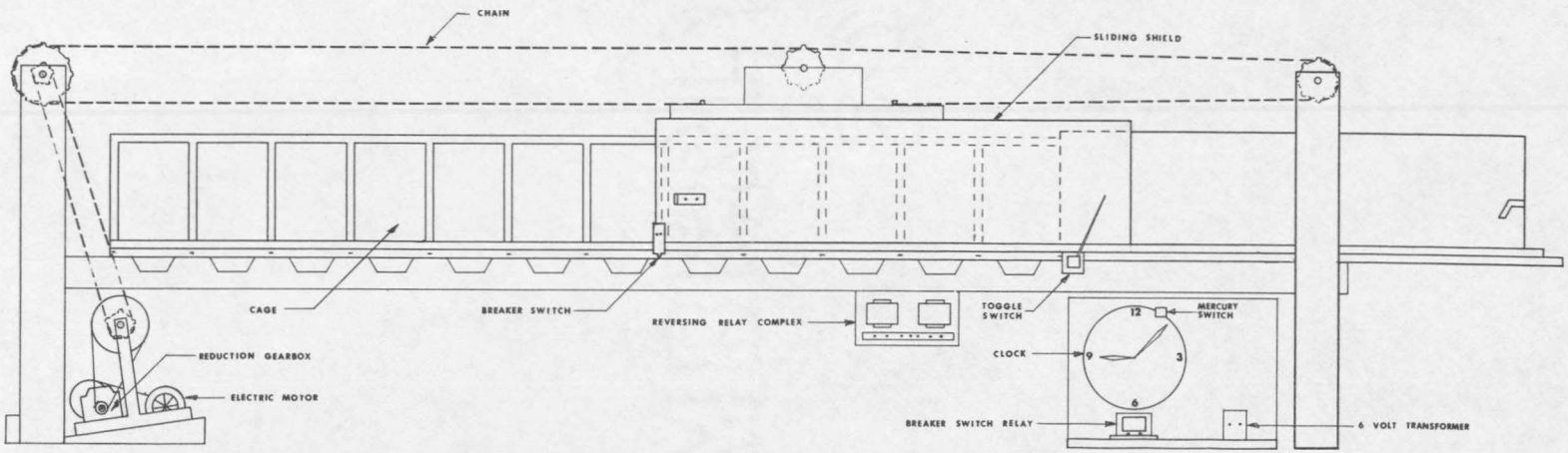


Figure 1. Schematic representation of the apparatus used to rear A. elliotti females under different photoperiods.

direction. It was necessary to connect a thermal time delay tube between the toggle switch and each of the relays which allowed the shield to remain at either end of the cage complex for one hour. If this was not done, the relay system would reverse the direction of the shield before it had come to a complete stop causing breakage of the chains or other moving parts. The relays were activated by a clock that tripped a mercury switch every hour sending a 20 second impulse of current to the motor causing the shield to cover or uncover a pair of cages depending on which direction it was moving.

The dimensions of each cage were 8" X 16" X 12". The top and one side of the cages were covered with fine screen. The pair of cages exposed to constant light was of the same dimensions and design but was not connected to the battery of cages with the sliding cover. A 5" X 9" hole was cut in the floor of each cage under which a baking pan of a similar size was attached. These pans were filled with soil taken from the same area where the grasshopper had been collected. Vials filled with fresh Western Wheatgrass were provided every other day.

Light for the experiment was provided by 16 Sylvania Gro-Lux fluorescent bulbs 96" in length. The fluorescent lights were supplemented with three 200 watt unfrosted incandescent bulbs. The light intensity derived from this system was found to be 52 candlepower when measured at the floor of the cages. The cages were placed in a room with no windows. Ventilation was provided by an exhaust fan. Due to the heat produced by the lights, the ambient diurnal temperature fluctuated only 2-3 degrees

from 84°F. The relative humidity averaged 17± 2% for the duration of the experiment.

Fifteen third and fourth instar nymphs were placed in each cage. Of these 15 grasshoppers ten were females and five were males. Those grasshoppers, males and females, which died during the first week of rearing were replaced. After the first week only males were replaced. Mortality for all grasshoppers after the first week was moderate. In only three cages did more than two grasshoppers per cage die during the experiment.

When the females emerged as adults they were marked with India ink and dated. Ovariectomies were performed on 127 females seven days after their imaginal moult. In most cases ten females were removed from each pair of cages having the same photoperiod. Two pairs of cages had a female mortality which did not permit a sample of this size. A total of nine females were removed from cages six and seven, and eight females were removed from cages 23 and 24.

The criterion used to determine the effect of a varied daylength on the rate of vitellogenesis was the length of the proximal oocyte. The length of the oocyte closest to the lateral oviduct for each ovariole was measured immediately after the ovaries were removed from the female. Those oocytes in which yolk was being resorbed were not measured, however, the number of oocyte resorptions was recorded for each female.

To examine the possible effect varied daylengths have upon frequency of oviposition, two pairs of adult grasshoppers were maintained in each of

the 26 cages for 28 days. Two females were picked at random from those females remaining in each cage after removing the necessary specimens for the previous experiment. In all cases the females used in this study were newly emerged and had not completed the first reproductive cycle.

After a period of four weeks the pans filled with soil were removed from the bottom of each cage and egg pods were separated from the soil by sifting through a 3/16 inch screen. The number of egg pods laid by the females in each cage was recorded as was the number of eggs within each pod.

Male grasshoppers were replaced in any cage in which there was mortality, whereas females were not. Upon death of a female the number of egg pods laid by both females was recorded immediately and then the remaining female was maintained until the end of the 28 day period.

RESULTS

Morphological and Histological Observations

The gross anatomy of the ovaries of adult females will be briefly described before entering into a detailed account of their postembryonic development and the process of vitellogenesis.

Each of the paired ovaries of A. elliotti consists of four to six elongate egg tubes or ovarioles (fig. 2). The typical number is five and only rarely are six ovarioles present. Distally, the ovarioles are drawn out into fine terminal filaments (fig. 3). The terminal filaments of the ovarioles from both ovaries unite to form a single suspensory ligament. The suspensory ligament attaches to the dorsal diaphragm in the mesothoracic region. Proximal to the terminal filament is the portion of the ovariole known as the germarium. The germarium contains oogonia, which are direct descendants of the primordial germ cells and prefollicular tissue. The prefollicular tissue later differentiates into the follicular epithelium that surrounds developing primary oocytes and also into the interfollicular tissue separating each follicle. Proximal to the germarium is the vitellarium. This region of the ovariole contains 15-17 successive egg compartments or follicles within which primary oocytes develop. The vitellarium of each ovariole contains an array of maturing follicles which represent a succession of physiological stages. It is in the vitellarium where primary oocytes undergo the greatest cytoplasmic growth and also where yolk deposition occurs. The proximal ends of the ovarioles are joined to the lateral oviduct in a serial fashion by means of structures known as stalks or pedicels (fig. 2).

The walls of the lateral oviducts are very elastic and are capable of considerable distension while holding mature eggs prior to fertilization and oviposition. An accessory gland is located at the apex of each lateral oviduct which provides materials used in the construction of the egg pod. Posteriorly, the lateral oviducts parallel either side of the gut and then turn ventrally to unite beneath the ventral nerve cord to form a single duct commonly referred to as the vagina.

The ovary of this species is of the panoistic type. Panoistic ovarioles, in contract to meroistic ovarioles, have no "nurse cells" which provide nutrients to the developing oocyte. Panoistic ovarioles are characteristic of most of the older orders such as Thysanura, Ephemeroptera, Odonata, Orthoptera, Isoptera and Plecoptera.

Ovarian development. Ovaries of newly hatched A. elliotti females are small paired structures lying dorsal to the midgut in the first to fourth abdominal segments. Each ovary is approximately 550 μ in length and 95 μ thick. The rudimentary ovarioles, when viewed in longitudinal section, appear as cellular columns, tapered at either end, and arranged in a single plane (figs. 4 and 5). Each ovariole is approximately 140 μ long and 50 μ thick. Unless otherwise indicated, ovariole length measurements do not include the terminal filament or the pedicel. Width measurements were taken at the widest portion of the ovariole. The morphogenesis of only one ovariole will be discussed for each nymphal stadium because all ovarioles of a specimen appear to develop in the same manner and rate.

PLATE I
(Figures 2-5)

Fig. 2. Freshly dissected ovaries from a gravid A. elliotti female. circa 15X.

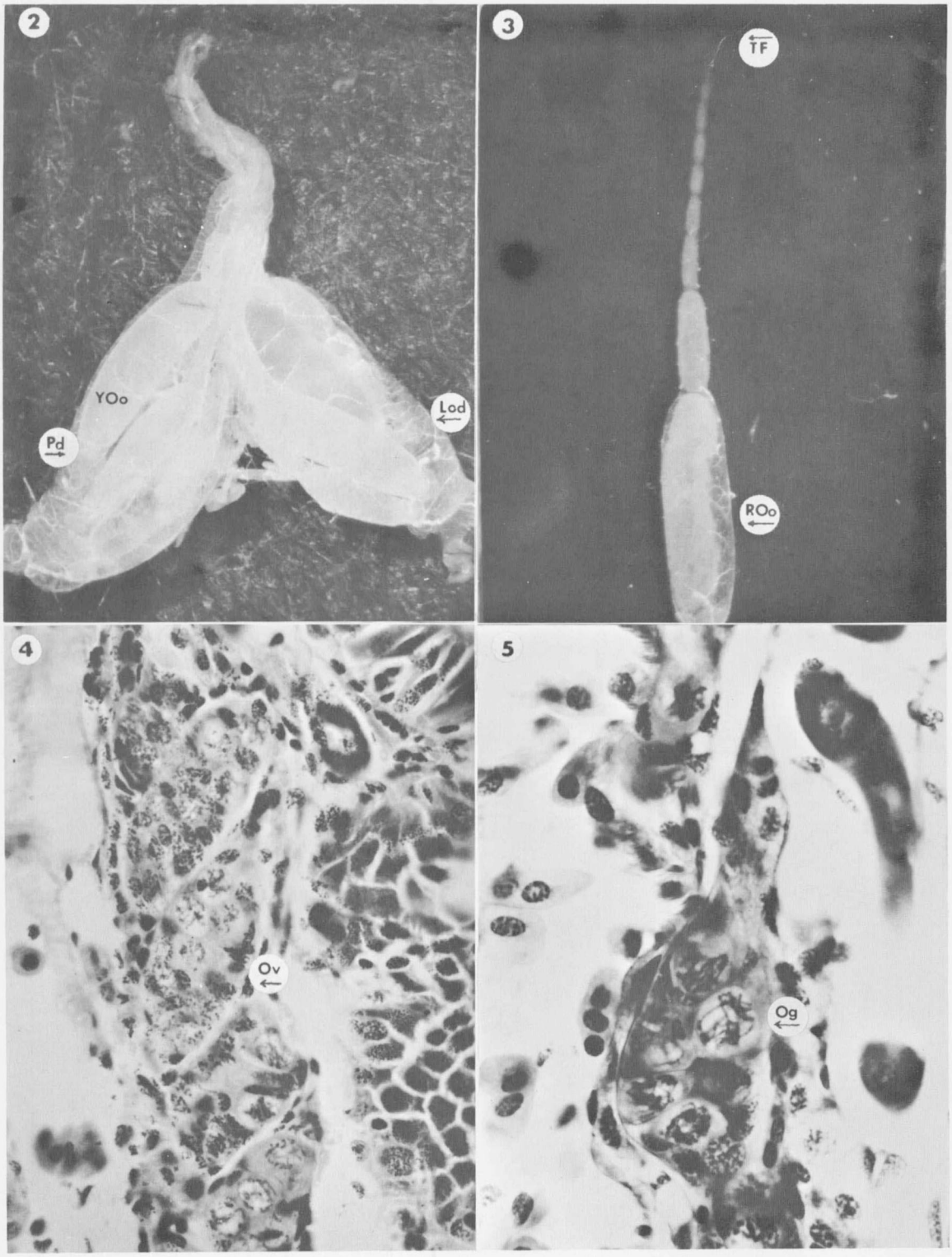
Fig. 3. Freshly dissected ovariole showing the succession of follicles. There is a resorbing oocyte in the proximal follicle. circa 15X.

Fig. 4. A longitudinal section of a first instar ovary from a newly hatched female which shows the serial arrangement of the four ovarioles. Sinha, Delafield's hematoxylin, 320X.

Fig. 5. Similar to the preceeding section but showing a single ovariole at a higher magnification. There are meiotic figures in the oogonia. 500X.

List of Abreviations

BD., basophilic droplets
Ch., chorion
DF., degenerating follicle
FE., follicular epithelium
Gg., gregarine
Gr., germarium
If., interfollicular tissue
IfZ., interfollicular zone
Lod., lateral oviduct
Oo., oocyte
Og., oogonium
Ov., ovariole
OS., ovariole sheath
Pd., pedicel
Pf., prefollicular tissue
PM., primordial muscle
ROo., resorbing oocyte
TF., terminal filament
Vc., vacuole
VCS., ventral cell strand
Yg., yolk granule
YOo., yolk-filled oocyte


2
YOo
Pd
Lod
3
TF
ROo
4
Ov
5
Og

The ovarioles of first instar nymphs are easily recognized. A large portion of the ovariole appears to be of a syncytial nature, wherein the nuclei of cells destined to form the various structural elements share a common cytoplasm. These nuclei, which are of mesodermal origin, are interspersed between the oogonia and form cone-shaped aggregations at either end of the ovariole. The distal group of nuclei tapers to a single strand, the terminal filament, which remains a syncytium for the life of the grasshopper (fig. 6). The proximal group of nuclei is part of the tissue which connects the ovariole to a ventral strand of cells (fig. 7) that is destined to become the lateral oviduct. This proximal aggregation of nuclei and the surrounding cytoplasm later loses its syncytial form and contributes to the cell structure of the pedicel. The follicular epithelium and interfollicular tissue of the more advanced instars are derived from the epithelial elements dispersed between the oogonia.

The nuclei of the oogonia are round, average 15 μ in diameter, and exhibit stages of the first meiotic prophase. Occasionally a mitotic figure is observed in the most distal of these cells. All germ cells within first instar ovarioles tend to have little cytoplasm and are closely packed.

Each ovariole is ensheathed by an outer membrane containing widely dispersed flattened nuclei and by a relatively thick non-cellular inner membrane, the tunica propria (fig. 8). Distally, the outer ovariole sheath becomes continuous with the terminal filament (fig. 9). Apposed

to the outer membrane the length of the ovariole are numerous tracheal endings.

The rudimentary lateral oviducts of newly hatched nymphs lie ventral and slightly lateral to each of the ovaries. The oviducal strand is appreciably thicker in the vicinity of the ovary than in the area paralleling the alimentary tract (figs. 10 and 11). The ovarioles abut directly onto this horizontal strand of cells (fig. 7) as the pedicel has not yet formed at this stage. Toward the end of the first instar the oviducal lumen begins to differentiate in the thicker portion of the strand in the region beneath the ovariole attachment, and progresses anteriorly and posteriorly from this point.

The ovarioles of second instar nymphs increase both in length and width. By the middle of the instar the average size of an ovariole is 820 μ long and 85 μ wide. The increase in length is due to growth and differentiation of the ovariole into two major zones, the germarium and the rudimentary vitellarium (figs. 12 and 13). The germarium of this stage makes up almost one-half of the total length of the ovariole. The germ cells have become more numerous due to cell division. A gradual increase in gonial size from the distal to proximal end of the germarium is apparent.

The division between the germarium and the rudimentary vitellarium is marked by a large increase in the size of the germ cells. The nuclei of these cells average 33 μ in diameter and the cytoplasm has augmented to such an extent that a single germ cell spans the width of the

PLATE II
(Figures 6-13)

Fig. 6. Distal end of mid-first instar ovariole showing the terminal filament. Sinha, Delafield's hematoxylin, 400X.

Fig. 7. Longitudinal section of first instar ovariole illustrating the posterior connection to the ventral cell strand at the time of hatching. Sinha, Delafield's hematoxylin, 400X.

Fig. 8. First instar ovariole showing tunica propria, outer ovariole sheath, and the meiotic figures within the oogonia. Sinha, Delafield's hematoxylin, 1280X.

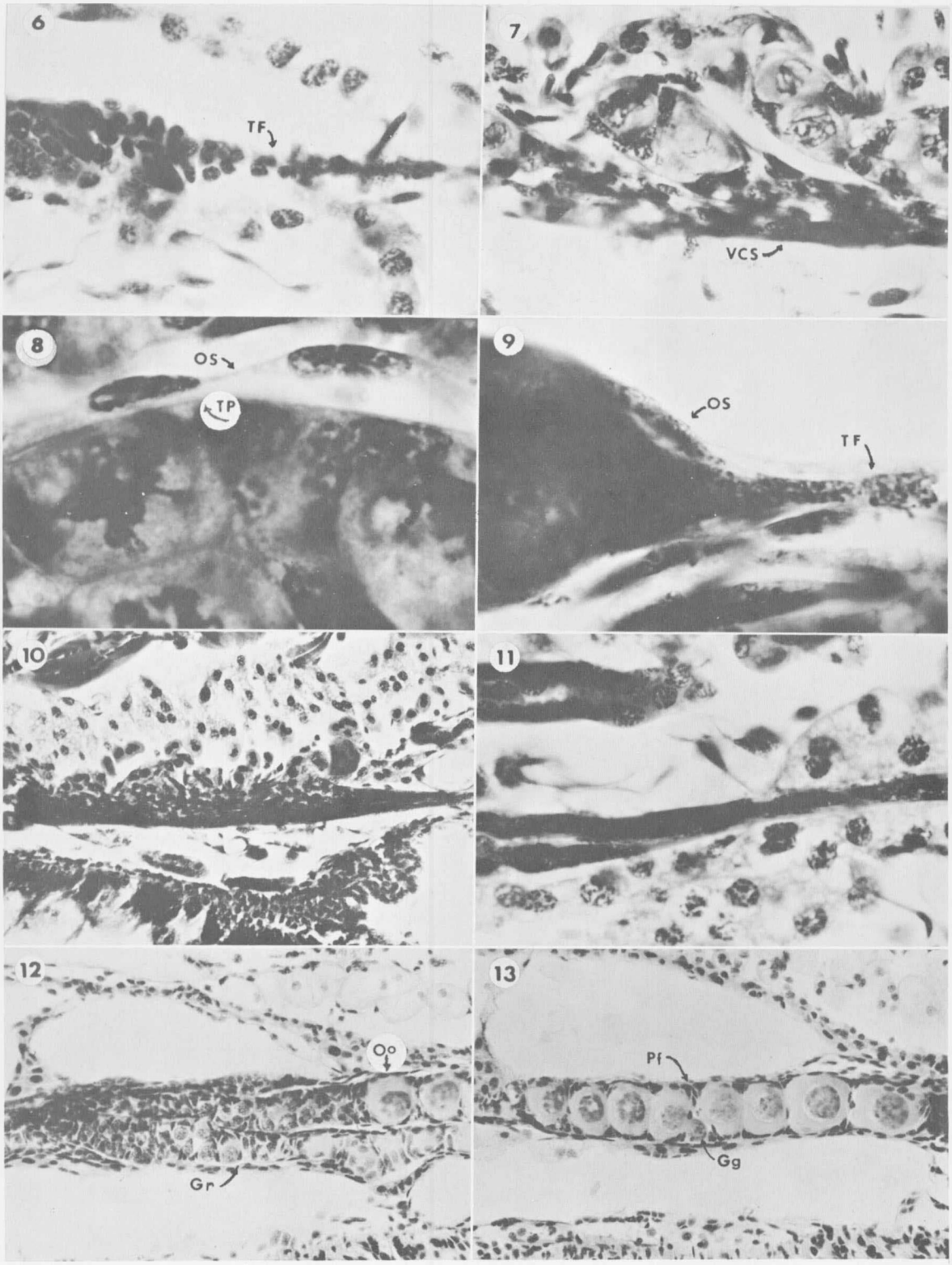
Fig. 9. Similar to above but showing a more anterior region of the ovariole. This photograph shows the extension of the outer ovariole sheath over the terminal filament. 1000X.

Fig. 10. Longitudinal section of a mid-first instar oviduct demonstrating the thick gonial region where the oviducal lumen begins to differentiate. Sinha, Delafield's hematoxylin, 140X.

Fig. 11. Similar to the above but this section shows a narrower portion of the oviduct posterior to the gonial region. The oviducal lumen is starting to form on the left. 430X.

Fig. 12. Sagittal section of two germaria from a mid-second instar female. The large primary oocyte marks the division between the germarium and rudimentary vitellarium. Sinha, Delafield's hematoxylin. 135X.

Fig. 13. Similar to above but shows the rudimentary vitellarium posterior to the region illustrated in the preceeding photograph. There is an encysted gregarine in the middle of this structure. 140X.


6
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rudimentary vitellarium.

All of the germ cells contained in the rudimentary vitellarium and a few of the most proximal germ cells of the germarium have nuclei in which the chromatin is in a diffuse condition (fig. 14). Individual chromosomes cannot be recognized in the nuclear reticulum of these cells. This nuclear condition appears to be a post-pachytene stage of the first maturation division. Thus, these cells are no longer oogonia, by definition, but are considered to be primary oocytes. Further meiotic division is not experienced by the primary oocytes until after they leave the vitellarium. During later instars and in the adult stage the nuclear network frequently becomes more concentrated in certain areas which yields a conformation similar to the "lampbrush chromosomes" of other animals (fig. 15). This concentration of chromatin material is usually more conspicuous during periods of extensive cytoplasmic growth.

The prefollicular nuclei of this stage are considerably more numerous in the germarium than in the rudimentary vitellarium. Mitotic activity is exhibited by many of the prefollicular nuclei throughout the length of the ovariole. Most of these nuclei in the rudimentary vitellarium are located at its periphery or are starting to form a layer between the primary oocytes and the ovariole sheaths (fig. 14). Toward the end of this instar the prefollicular tissue invades the area between one or two of the most proximal oocytes and completely surrounds them (fig. 13). This layer is not yet definitive follicular epithelium as no cellular membranes can be discerned at this time.

As the length of the ovarioles increases, the typical slanted serial arrangement present in the first instar ovary is lost. Instead, the ovarioles now lie parallel to the longitudinal plane of the grasshopper and are depressed between the dorsal diaphragm and the midgut. As shown in Figure 16 this lengthening is accompanied by a change in the position of the ovarioles in relation to each other. Generally, the trend is for the ovarioles to spread laterally and also, for some of them to become situated dorsal to their counterparts. This increase in length is likewise associated with a lateral movement of the proximal ends of the ovarioles which moves the oviduct in the gonadal region from its mostly dorsal position to the definitive lateral position.

By the end of the second instar a lumen is present from the distal end of the lateral oviduct to where it joins the vaginal duct. The oviducal wall of this stage has three layers, one of which is rather indistinct (fig. 17). There is an inner lining of columnar epithelium and an outer connective tissue sheath which appears to be continuous with the outer ovariole sheath. Located between the epithelial layer and the connective tissue sheath are some widely distributed elongate cells. These cells are presumptive muscle cells which later differentiate into muscle tissue in the young adult.

The pedicel differentiates during the second instar as an evagination of the lateral oviduct at the site where it connects to the ovariole. This evagination later lengthens to form a short tube which is continuous with the lumen of the oviduct (fig. 18).

About the middle of the third instar the ovarioles average 1000 μ (1 millimeter) in length and 100 μ in width. There has been little change from the morphology of second instar ovarioles except for the increase in size.

Cell division has increased the number of oogonia over that of the preceeding instar. By division and an increase in cytoplasm of the cells forming the outer ovariole sheath and an increase in mass for the tunica propria, the enveloping tissues are able to keep pace with the expansion of the ovarioles.

The more proximal primary oocytes continue to increase in cytoplasmic volume to where length exceeds the width of these cells (fig. 19.). The average dimensions of the most proximal primary oocyte are 125 μ long and 25 μ in diameter. The nucleus of this cell has an ovoid shape in contrast to the rounded nuclei of the more distal oocytes. Because the pre-follicular tissue surrounding the proximal primary oocytes does not increase in mass in proportion to the growth of the oocytes, the pre-follicular nuclei become quite flattened in appearance (fig. 20).

The lateral oviducts and pedicels continue to increase both in length and width during the third instar.

The fourth instar ovariole has increased about 200 μ in length by the middle of the instar, whereas the width remains near that of the preceeding stage. The augmentation in length appears to arise from the addition of primary oocytes at the distal end of the rudimentary vitellarium. These cells become quite crowded and compact to the point where cell width

PLATE III
(Figures 14-21)

Fig. 14. Proximal primary oocyte of a late second instar ovariole. The nuclear material within the oocyte nucleus is in a diffuse condition. Prefollicular tissue is beginning to surround the oocyte. Sinha, Delafield's hematoxylin, 650X.

Fig. 15. Oocyte nucleus of a fifth instar proximal oocyte illustrating isolated concentrations of chromatin material. Ammerman, Mallory's triple stain, 575X.

Fig. 16. Transverse section of a second instar female showing the tiered arrangement of the ovarioles. Sinha, Delafield's hematoxylin, 135X.

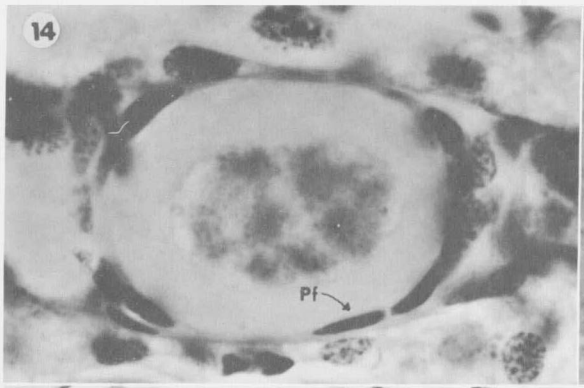
Fig. 17. Section of a second instar oviduct displaying the three cell layers making up the oviducal wall. Sinha, Delafield's hematoxylin, 1000X.

Fig. 18. Similar to the above but at a lower magnification showing pedicel formation by evagination of the oviducal wall. 200X.

Fig. 19. Longitudinal section of a portion of a mid-third instar vitellarium illustrating the lengthening of the primary oocytes. Sinha, Delafield's hematoxylin, 120X.

Fig. 20. Proximal oocyte of a late third instar ovariole. The nuclei in the surrounding prefollicular tissue are flattened. Sinha, Delafield's hematoxylin, 450X.

Fig. 21. Longitudinal section of a mid-fourth instar ovariole showing the compacting of primary oocytes near the distal end of the vitellarium. Sinha, Delafield's hematoxylin, 165X.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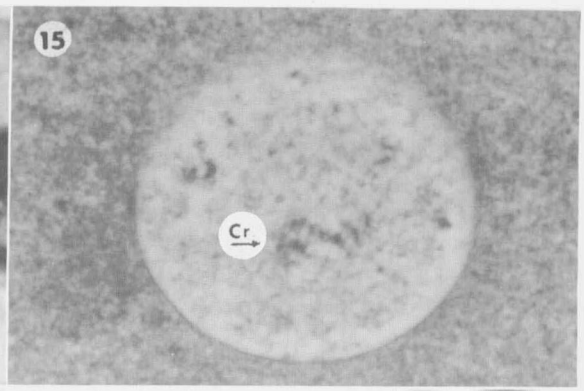
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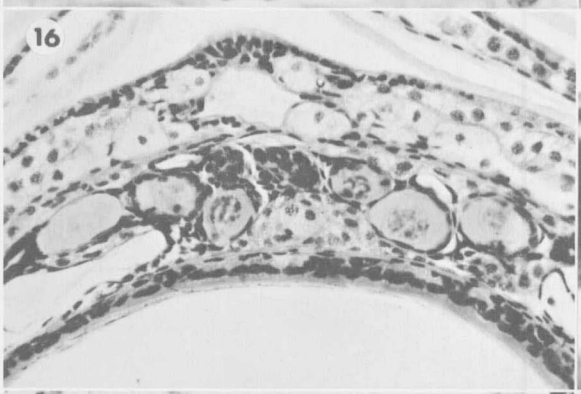
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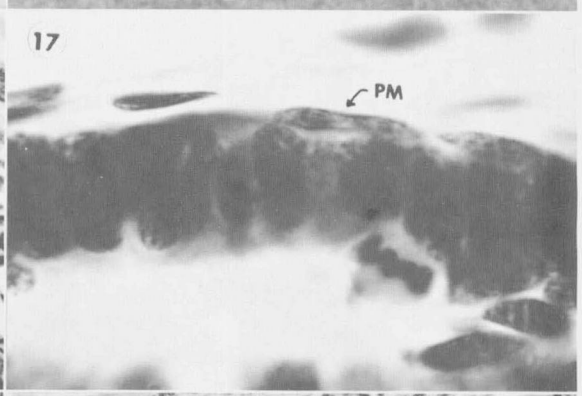
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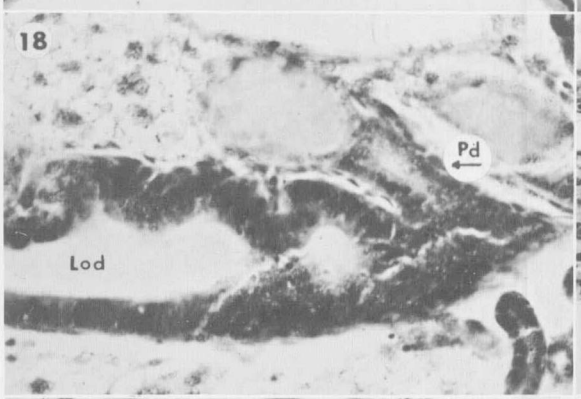
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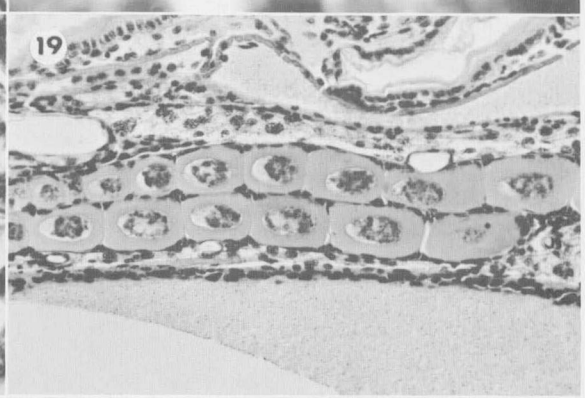
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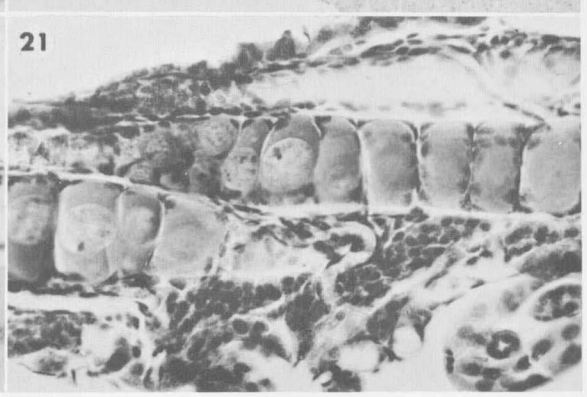
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exceeds length (fig. 21). This is in direct contrast to the dimensions of those primary oocytes at the proximal end of the rudimentary vitellarium.

During this instar the regions between the last two or three primary oocytes at the proximal end of the rudimentary vitellarium differentiate into interfollicular zones. The proximal primary oocyte of each ovariole and its surrounding layer of prefollicular tissue becomes separated from the oviducal cells forming the pedicel. This separation is due to a proliferation of transversely arranged prefollicular tissue between the pedicel and the oocyte (fig. 22). Similar zones also form between the primary oocytes immediately distal to the proximal oocyte.

By the middle of the fifth instar the ovarioles are almost three times as long as those midway in the preceeding stage. The ovarioles now average 3200 μ (3.2 millimeters) in length and 135 μ in width. The increase in length is accompanied by a considerable gain of cytoplasm by the primary oocytes. For example, the proximal primary oocyte at this stage of development averages 365 μ long and 110 μ in diameter.

It is during the fifth instar that the prefollicular tissue surrounding primary oocytes, in which yolk is to be deposited first, begins to assume its cellular form. An intense amount of mitotic activity results in the encasement of the older primary oocytes by a layer of tissue with many nuclei and little cytoplasm (fig. 23). During the latter portion of this instar this tissue loses its syncytial condition to become a single layer of cuboidal cells. Although by definition this layer is not a true epithelium, it is generally referred to as the follicular epithelium.

There has been little noticeable histological change in the enveloping membranes except for an increase in the number of nuclei present per unit surface area in the outer ovariole sheath.

At the posterior end of the proximal oocytes a number of basophilic droplets were observed (fig. 24). These droplets were noticed only in fifth instar oocytes. Neither the origin nor the significance of these structures could be determined.

The interfollicular tissue has reached its definitive state in the interfollicular zones which serially separate the last three primary oocytes at the proximal end of the ovariole. There is little mitotic activity within these zones after the midpoint of the instar. Anterior to the fully formed interfollicular zones one can still observe the formation of similar separating structures between the younger primary oocytes. The nuclei of interfollicular tissue retain an appearance like that of prefollicular tissue from which it was derived. It is readily noticeable that interfollicular tissue is cytologically distinct from definitive follicular epithelium (fig. 25).

There is a prodigious amount of mitotic activity occurring in the epithelial lining of the oviducts during this instar. The cells become compacted and assume a stratified appearance (fig. 26), although this stratification is not evident in the mature oviducts. The pedicel again increases in length as in the preceeding instars. Its length is approximately 3/4 the length of the adjacent primary oocyte and the cell structure is very similar to that of the lateral oviduct.

The ovarioles of newly moulted adult females are not significantly different from those of the latter part of the fifth instar. However, 2-3 days after the moult the ovarioles begin a period of rapid growth resulting in great increase in size. This tremendous increase in size usually occurs in a span of 7-9 days and appears to be largely due to deposition of yolk in the last and penultimate oocytes. A smaller portion of ovariole enlargement is associated with cytoplasmic augmentation in the other primary oocytes.

The ovariole averages 15.5 mm long and 1.4 mm wide by the time yolk deposition has been completed in the proximal primary oocyte. Of the total mean length of 15.5 mm, the last follicle contributes an average of 6.0 mm and the penultimate, 2.9 mm.

The cuboidal epithelium surrounding the proximal primary oocyte in the fifth instar has so increased in cellular number that it has become a columnar epithelium preliminary to yolk deposition in the early adult stage (fig. 27). The cuboidal cells have divided and grown with such rapidity that they have become compressed into the columnar form. It is only toward the end of vitellogenesis for this particular follicle that most of the epithelial cells lose their columnar shape. Cellular division in the epithelial layer terminates but oocyte expansion does not, consequently, the cells are stretched to such an extent that cell width now exceeds cell height (fig. 28). It is when the cells have this form that they produce a non-chitinous covering, the chorion, around the oocyte after yolk deposition is completed.

It is also during the early adult stage that prefollicular tissue surrounding 4-5 primary oocytes immediately distal to the proximal oocyte differentiates into a cuboidal epithelium. All during the period that the female is reproductively active there are 4-5 primary oocytes encased by a layer of follicle cells besides the proximal oocyte. As soon as a proximal primary oocyte leaves the ovariole or is resorbed, the prefollicular tissue differentiates around the primary oocyte immediately distal to the four or five formed follicles.

As indicated above, not all cells of a follicular epithelium encasing an oocyte at the end of vitellogenesis have the same form. There is further differentiation of a relatively small number of epithelial cells forming a cap at the posterior end of the follicle (fig. 29). These cells are larger than the other follicle cells and are mostly of a columnar form. This aggregation of cells is responsible for secreting the specialized layers of the chorion which is part of the hydropyle apparatus. The hydropyle functions in the uptake of water in the mature egg.

Although movement of the nucleus from its central position is noticeable in the proximal oocyte of a fifth instar ovariole, it is during the early adult stage that additional oocytes at the proximal end of the ovariole acquire a definite polarity (fig. 30). The oocyte nucleus becomes located at the proximal end of the cell and only rarely does it become situated at the distal end of the cell. The nuclei are spherical and average 80 μ in diameter. The chromatin material remains in the

PLATE IV
(Figures 22-29)

Fig. 22. Proximal end of an early fourth instar ovariole illustrating the invasion of interfollicular tissue between the pedicel and the proximal follicle. Sinha, Delafield's hematoxylin, 1000X.

Fig. 23. Two layers of prefollicular tissue surrounding adjacent mid-fifth instar oocytes. This tissue is beginning to form cellular divisions. Sinha, Delafield's hematoxylin, 1000X.

Fig. 24. Posterior end of a late fifth instar proximal oocyte displaying basophilic droplets and peripheral vacuoles. Sinha, Delafield's hematoxylin, 400X.

Fig. 25. Interfollicular zone between two successive fifth instar primary oocytes. Sinha, Delafield's hematoxylin, 510X.

Fig. 26. Proximal oocyte pedicel and lateral oviduct of a late fifth instar female. Sinha, Delafield's hematoxylin, 100X.

Fig. 27. Columnar follicular epithelium surrounding a pre-vitellogenic adult primary oocyte. Ammerman, Mallory's triple stain, 400X.

Fig. 28. Section of the follicle epithelium showing two elongate follicle cells during the latter stages of yolk deposition in the proximal oocyte. Carnoy, Gallocyanin-chrome alum, 1000X.

Fig. 29. Posterior end of a proximal follicle illustrating the specialized follicle cells that secrete the chorion above the hydropyle. Carnoy, Gallocyanin-chrome alum, 100X.