THE UNIVERSITY OF MANITOBA

Structural and Physiological Aspects of 5th instar Ovarian Development in *Rhodnius prolixus* (Insecta: Hemiptera)

by

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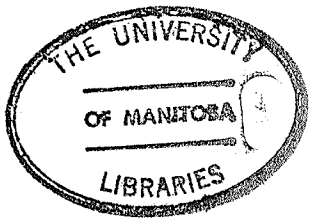

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ABSTRACT

Differentiation events accompanying the larval-adult ovarian transformation in Rhodnius prolixus can be divided into three phases: proliferative phase (unfed-8 days post feed), early differentiation phase (9 DPF-15 DPF) and late differentiation phase (16 DPF-moult at 21 DPF). Ovarioles remain morphologically larval until feeding initiates development. The unfed ovariole contains germ cells surrounding a central trophic core region with the "germarial lumen" occupying the basal region of the tropharium immediately above the prefollicular tissue. Utilizing various light and electron microscopical techniques, coupled with autoradiography, the timing and morphological details of germ cell differentiation as well as mesodermal and structural differentiation were determined. Mitosis of germ cells during the proliferative phase results in a progressive increase in tropharial size with no differentiation of tissues. Regional specialization within the ovariole marks the beginning of the early differentiation phase. A zone of oocytes is established at the base of the tropharium with nuclei containing synaptonemal complexes and condensing chromosomes. Nurse cell differentiation is characterized by nucleolar elaboration and nucleo-cytoplasmic transport, the cytoplasm becoming rich in ribosomes.

Autoradiographic results suggest that physiological nurse cell - oocyte divergence occurs concurrently with morphological divergence. Prefollicular tissue is divided into apical and basal zones with apical zone differentiation occurring during early and late differentiation phases. Intercellular bridges, derived from incomplete cytokinesis, interconnect nurse cells,

oocytes, and connect nurse cells to oocytes. Larval trophic core consists of a tortuous arrangement of cell processes. Massive membrane fusions occur in the core during the late differentiation phase, transforming this area to its adult structure. Birefringent core and cords are visible by 18 DPF. During the late differentiation phase, oocytes initiate previtellogenic growth and accumulate material from tropharium restructuring. Results from ovarian transplant experiments indicate that the presence of ecdysone initiates germ cell differentiation. An absence of JH in the 5th instar is necessary for normal differentiation of normal mesodermal components and adult ovarian structure.

INTRODUCTION

Cells interact in a variety of ways such as aggregation and adhesion, contact inhibition, electrical communication, passage of molecules or ions between cells and hormonal interactions (Deuchar, 1975). These interactions, which may be either unidirectional or reciprocal, cause a structural and/or functional change in the cells involved. Research on cell interactions, although undertaken since the first description of cells, has increased in the past 20 years. Recent advances in biochemical and microscopical technology have allowed workers from a variety of scientific disciplines to examine, in far greater detail than before, the diversities and complexities of interacting cell systems. Some researchers have utilized fully differentiated adult tissues in their investigations while others have used established tissue culture cell lines. Still others have investigated a variety of cell interactions in numerous developmental processes. Information from these latter studies has added to the understanding not only of the structural and chemical nature of the interaction but also of the dynamic nature of these interactions and their relationship with many developmental processes.

The growth of an oocyte involves a complex series of events during which the oocyte is intimately associated with one or more types of accessory cells. Oocytes within vertebrate ovaries develop in conjunction with a follicular epithelium of

varying layers. In some invertebrate groups, developing oocytes associate with two cell types: follicle cells and nurse cells. This type of cell interaction is especially well developed in insects possessing the meroistic ovary type (Telfer, 1975).

The degree of complexity and specialization of these cell interactions is truly remarkable and has provided a unique opportunity for analysis of basic developmental problems. Insights into the development of oocytes, storage of various RNA's, physiological controls and numerous other aspects have been obtained by the experimental exploitation of the meroistic system (see Davidson, 1976).

Of the two cell types that interact with the oocyte, nurse cells provide the developing oocytes with a significant portion of their structural and metabolic machinery. Mesodermally derived follicle cells enveloping the egg are involved in regulation of materials passing to the egg, in a nutritive function, and in secondary egg coat formation (Longo and Anderson, 1974). Both nurse cells and oocytes originate from the same stem cell line and retain, until late oogenesis, a syncytial association via intercellular bridges through which the nurse cell passes synthetic products to the oocyte (Telfer, 1975).

Two structurally different ovarian classes exist within the meroistic category: polytrophic and telotrophic types. Polytrophic ovarioles, the ovariole being the functional unit of the ovary, consist of two regions: a germarium region

containing oogonia and forming ovarian follicles, and a vitellarium containing completed ovarian follicles at later stages of development. Each follicle, surrounded by a follicular epithelium, consists of a fixed number of nurse cells (which varies with species) interconnected and connected to a single oocyte by intercellular bridges (Bonhag, 1958). Telotrophic ovarioles consist of a lanceolate germarium containing apical nurse cells surrounding a central trophic core region. Oocytes and prefollicular tissue occupy the basal region of the germarium and of the vitellarium. Oocytes are connected to the trophic core by cytoplasmic projections, the nutritive or trophic cords (Bonhag, 1958). Although structural differences distinguish these two ovarian types, they have certain fundamental similarities in their cell interactions. While the focus of this study is the telotrophic system, background information available on the polytrophic system can possibly provide clues to assist interpretation of the nurse cell and follicle cell - oocyte interactions in the telotrophic ovary. Parallels may exist in germ cell proliferation, oocyte determination, intercellular bridge formation, follicle formation and other ovarian developmental processes. For background information on a morphologically simpler system, the following section reviews the polytrophic situation.

Polytrophic Ovarian Development.

Structure and function of the polytrophic - meroistic ovary have been extensively explored. Many of these studies

have focussed on the events of follicle development from an oogonium to the initiation of previtellogenesis, often concentrating on determination of the precise geometric arrangement of the nurse cell - oocyte complex and on morphological and physiological events involved in nurse cell - oocyte divergence.

Brown and King (1964) and later Koch and King (1966), indicate that in Drosophila, oogonia, restricted to the apex of the germarium, serve as a stem cell population. Mitosis produces two daughter cells, one of which remains at the apex of the germarium as an oogonium. The other daughter cell, or cystoblast leaves this region and commences a series of four mitoses producing a 16 cystocyte unit. Cystocytes remain interconnected by intercellular bridges formed by incomplete cytokinesis. Differentiation of the 16 cystocytes results in 15 nurse cells and one oocyte.

The precise geometric arrangement of the nurse cell - oocyte complex is known for four cases: Macrothylacia (Hirschler, 1955) and Hyalophora (King and Aggarwal, 1965) each containing eight cell units, and Dytiscus (Giardina, 1901) and Drosophila (Koch and King, 1966) each containing 16 cell units. Physiological evidence indicates that there are two phases of cystocyte complex development. The first phase is synchronous mitosis within all cystocytes (Chandley, 1966). Following completion of mitosis, the asynchronous endomitotic phase commences, accompanying nurse cell - oocyte divergence (King and Burnett, 1959; Urbani, 1970). Asynchronous endomitotic

chromosome replications, occurring within nurse cell nuclei, are related to their role in copious RNA production.

As a result of morphological observations, early investigators attributed a nutritive function to nurse cells (Wilson, 1925). More recent molecular biological evidence confirmed some of these interpretations. Using autoradiography, Bier (1963) clearly demonstrated that polytrophic nurse cells function primarily to produce RNA for subsequent transport to the oocyte. At least two variations exist in the timing of this bulk transport within polytrophic systems. Bier (1963) reported that most RNA accumulates within nurse cell cytoplasm until the final stages of follicular oocyte growth in Musca. This stored RNA, along with the nurse cell cytoplasm, is injected into the oocyte at the last phase of follicle growth. In Hyalophora, nurse cell - produced RNA is transported to the oocyte constantly during its growth, and terminal injection of nurse cell cytoplasm plays only a minor role (Pollack and Telfer, 1969). In addition to RNA, ribosomes and proteins are transported to the oocyte. Mahowald and Strassheim (1970) have reported the transport of centrioles from Drosophila nurse cells. Many authors have suggested from static observations that nurse cell produce and export mitochondria to the oocyte, although appropriate labelling and tracing experiments have yet to be done (Telfer, 1975).

Although the asynchrony of nurse cell chromosome endomitotic replications accompanies nurse cell - oocyte divergence, this

does not signify a causal relationship. Various polytrophic ovary investigators have speculated on the cause or causes of nurse cell - oocyte divergence. Giardina (1901) proposed that the extra-chromosomal chromatin body of Dytiscus oocytes was the oocyte determinant since, through four mitotic divisions, it resided in the cytoplasm of the cystocyte which ultimately became the oocyte. However, examination of other polytrophic systems by Giardina and others revealed that the extra-chromosomal chromatin body was unique to Dytiscus and one other genus. Hirschler (1955) studying Macrothylacia postulated trophic mechanism for oocyte differentiation. Since the oocyte formed from one of the two cells with three bridges, it was one of the best fed cystocytes. Hirschler's theory provides no explanation of the way in which the polarity of nutrient flow would be established. Woodruff and Telfer's (1973) demonstration, in cecropia, of an electrical gradient establishing directional nurse cell - oocyte flow suggests that a re-examination of Hirschler's theory is required.

The most widely accepted theory of oocyte determination in polytrophic systems is that of Koch et al. (1967). They suggested that cortical cytoplasm associated with the first formed intercellular bridge is responsible for oocyte determination. Supporting evidence was the observation that the nurse cell adjacent to the oocyte and possessing four intercellular bridges forms synaptonemal complexes. Synaptonemal

complex formation indicates that this nurse cell is involved in a program of oocyte development. This model is consistent with all other polytrophic systems investigated, in that the oocyte determinant is always associated somehow with the first-formed intercellular bridge (Telfer, 1975). Of the two cells containing synaptonemal complexes, the first to interact with prefollicular tissue became the oocyte. Subsequently, synaptonemal complexes within the other cell nucleus break down and a nurse cell pattern of differentiation ensues (Koch et al., 1967).

Extensive genetic knowledge has permitted King and co-workers to analyze differentiation events within the Drosophila ovary (Koch and King, 1964; Smith and King, 1968; Klug, Bodenstein and King, 1968; King and Hewlett, 1970; Klug et al., 1970; King, 1972; Johnson and King, 1972). The recessive mutations, fused (fu, X - 59.5) and female sterile (fes, 2 - 5), produce ovarian tumors causing sterility. Findings indicated that fu and fes gene products may stabilize intercellular bridges. In addition, the authors concluded that cystocytes are programmed to enter nurse cell differentiation pathway upon completion of mitosis. Studies of the crossover suppressor of Gowen (c (3) G, 3 - 58) revealed that formation of synaptonemal complexes is required for meiotic crossing-over while the normal C (3) G gene probably codes for an essential component of zygosomes, the protein component of the complex.

Several mutations affecting nurse cell nuclear function have also been identified. Studies of these mutants provided insights into the necessary series of events for normal nurse cell nuclear function.

Despite extensive research on hormonal control of morphogenesis, few studies have directly investigated hormonal control of oogonial transformation and early ovarial differentiation. King (1975), working on the control of polytrophic ovarian development, summarized the work of earlier German investigators, integrating their results with his findings. This compilation indicated that a pulse of ecdysone from the prepupal prothoracic gland programs the germ cells to undergo incomplete cytokinesis. The phenomenon of division with incomplete cytokinesis is presumably transmitted to daughter cells since it continues into the adult after prothoracic gland degeneration. Since only oogonia in the apical germarium undergo complete proliferative mitoses, King postulated an interaction of terminal filament cells and oogonia permitting completion of cytokinesis. Daughter cells removed from the terminal influence follow the incomplete cytokinetic pathway.

Telotrophic Structure and Function

With the information from the differentiation of the relatively simple cystocyte unit with its small cell number, one can now analyze more clearly the architecturally more

complex telotrophic situation. Telotrophic ovaries, as described earlier, are found in the polyphagous Coleoptera and the Heteroptera. Polyphagous coleopteran ovaries, in contrast to heteropteran ovaries, lack the central trophic core region within the tropharium. Buning (1972) proposed that this difference is sufficient to postulate independent origin of telotrophic structure within these two groups. However, while noting this important difference, I will, for convenience and discussional purposes, consider both ovariole types together.

A number of studies of adult ovarian structure and vitellogenesis has provided understanding, at both light and electron microscopic levels, of adult structure and of cell interactions involved in egg production (Bonhag and Wick, 1953; Bonhag, 1955a and b; Eschenberg and Dunlap, 1966; Patchen and Davey, 1968; Brunt, 1971; Huebner and Anderson, 1972a,b,c; Ullmann, 1973; Schreiner, 1977a and b). Histochemical, biochemical and autoradiographic techniques have elucidated nurse cell function in telotrophic systems (Bonhag, 1955a and b; Anderson and Beams, 1956; Vanderberg, 1963; Zinsmeister and Davenport, 1971; Mays, 1972; Ullmann, 1973; Cave, 1975). As in polytrophic ovaries, telotrophic nurse cells produce RNA for transport to the oocyte. Endomitotic polyploidization of the entire nurse cell genome, rather than amplification of the ribosomal RNA genes, enhances synthesis of RNA (Cave, 1975). Controversy still surrounds the transport of DNA to the oocyte suggested by Vanderberg (1963) and refuted by Zinsmeister and Davenport (1971). Although protein synthesis can be demonstrated

in the trophic core and cords, the sequential transport of newly synthesized proteins from nurse cells to oocytes is not likely (Zinsmeister and Davenport, 1971). Transport of RNA through the core and cords is essentially unidirectional (Macgregor and Stebbings, 1970). Mays (1972) suggested slow and fast transport rates within the cords reminiscent of axoplasmic flow in neurons (Kreutzberg, 1969). The bulk of RNA, likely the ribosomal component, is transported slowly, while a smaller fraction of transported RNA, thought to be m-RNA, utilizes a faster route. In hemipteran ovaries, the transport route, via the trophic core and cords, contains longitudinally oriented microtubules (Hamon and Folliot, 1969; Brunt, 1971; Huebner and Anderson, 1970; Macgregor and Stebbings, 1970). The microtubules' role in structural maintenance and transport has been verified (Huebner and Anderson, 1970; Macgregor and Stebbings, 1970) although their contribution to motive force generation is yet unclear. Hyams and Stebbings (1977) have experimented on possible transport mechanisms.

While adult structure and function have received much attention, the larval development of telotrophic structure and various cell interrelationships within the ovariole have not been investigated as extensively, and much confusion yet exists about some aspects (polyphage Coleoptera - Buning, 1972, 1978; Ullmann, 1973; Kloc and Matuszewski, 1977. Heteroptera - Wick and Bonhag, 1955; Masner, 1968; Case, 1970; Huebner and Anderson,

1972c; Choi and Nagl, 1976, 1977). This confusion stems from limitations of technique, for example light microscope resolution as compared to the much greater resolution of electron microscopy, and from interpretational problems arising from presentation of weak data leading to deceptive conclusions. Failure to correlate changes to a developmental time frame is a limiting factor in many of these studies. The need for establishment of a reproducible time frame for accurate comparisons was recognized by Buning (1978).

A survey of literature reveals a need for integrated examination of telotrophic ovarian development. This approach would aid both the understanding of sibling relationship ontogeny and the clearer understanding of adult structure and function. In addition, more complete information would provide the basis for both further polytrophic - telotrophic comparisons and further study in cell-to-cell interactions during development in a system which has a unique and exploitable architectural organization.

The objective of this study, then, is to apply an integrated approach to the investigation of 5th instar ovarian development in the reduviid hemipteran, Rhodnius prolixus. Rhodnius was chosen because of the availability of detailed information on both its adult ovarian structure (Patchen and Davey, 1968; Huebner and Anderson, 1972 a,b,c,) and its reproductive physiology (Wigglesworth, 1948; Davey, 1967; Pratt and Davey, 1972 a,b,c,; Huebner and Davey, 1973). Some information is available on the ultrastructure of 5th instar ovarian nurse cells, oocytes,

prefollicular cells, trophic core and cell interactions (Huebner and Anderson, 1972 a,b,c). Although data from these studies indicated that fundamental differentiation events, as well as changes in cell interactions, occur during this stage, details of timing and progression of differentiation events were not determined. Developmental events for each instar and egg production in the adult are initiated by a blood meal, allowing reproducible timing of events related to feeding times. This investigation was restricted to the 5th instar as the major larval-adult ovarian transformation events take place then. The specific objectives of this study were four-fold:

1. To establish a developmental framework of the ovary during the 5th instar.
2. To investigate nurse cell - oocyte morphological divergence.
3. To study the various cell interactions within the ovary which accompany the larval-adult transformation and to determine the sequence and duration of each.
4. To investigate endocrine aspects of ovarian differentiation.

In order to accomplish these goals, I have utilized observations on live preparations, light and electron microscopical techniques coupled with autoradiography on fixed specimens, and ovarian transplant techniques.

MATERIALS AND METHODS

Rearing and Handling

Animals were kept in darkness in a Controlled Environments Inc. incubator at 27°C with 70% R.H.. Wide mouth jars with wire mesh lids were used to house approximately 20 adults or an equivalent number of instars. Within each jar, a piece of fluted filter paper served for urine absorption and as an egg laying substrate. The colony was separated into breeding stock, female virgins and experimentals. Males, upon reaching the 5th instar, were separated from females. The breeding colony was fed periodically upon the shaven bellies of rabbits. Instars and experimentals were fed as required.

Dissection Technique

Ovaries were removed from animals with a combination of jeweller's forceps, iris scissors, microscalpels, insect pins and microhooks (made from tungsten wire by electrolytic etching, Hubel, 1957). All dissections were performed using a Wild M3 or M5 dissecting microscope. Insects were immobilized by removing their legs and pinning them through the thorax in a petri dish lined with Sylgard* (Dow Corning Corp.). After removing the dorsal cuticle covering the abdomen, the animal was bathed in either Grace's Insect Culture Medium (Grand Island Biological Supply Co.), Yeager's saline or Roach Heart Ringers minus dextran (Lockwood, 1961) for live observations.

* Dow Corning Corporation, Midland, Mich. U.S.A 48640

Live Observations

To supplement fixed and sectioned material, stages of 5th instar ovarian development were studied in live tissue using interference and polarizing microscopy. Examination of ovaries by interference microscopy was performed at two day intervals from the unfed state to 8 DPF (days post feed) and from 16 DPF until the adult moult (21 DPF). During the interval 8 DPF to 16 DPF, ovaries were examined daily. Ovaries taken at daily intervals from 16 DPF until the adult moult were used in the polarizing microscopy investigations. The ovaries were placed within a small drop of medium on a clean microscope slide. Vaseline applied to the corners of the coverslip prevented uncontrolled flattening and/or rupture of the preparation by supporting the coverslip during viewing. A Zeiss Photomicroscope II equipped with Nomarski differential interference optics and with a polarizer and analyzer was used in these investigations.

Fixation and Embedding

Ovaries were fixed and embedded at daily intervals during the instar from the unfed state to the adult moult (21 DPF). Animals were dissected and ovaries were removed in fixative. Two fixatives were used in this study: a modified Karnovsky's (Karnovsky, 1965) containing 3% glutaraldehyde, 1% paraformaldehyde and 0.05% $CaCl_2$ in 0.1 M sodium cacodylate buffer pH 7.2 and a microtubule stabilizing fixative (Luftig et al., 1977)

containing 5% glutaraldehyde, 1 mM $MgSO_4$, 1 mM GTP, 2 mM ethylene glycol bis (β - aminoethyl ether) - N - N' - tetracetic acid (EGTA) in 100 mM PIPES buffer pH 6.9. Tissues were fixed for 1 - 1½ hours. After fixation, ovaries were washed in 0.1 M sodium cacodylate buffer pH 7.2 for at least 1 hour and were subsequently post-fixed for 1 hour in 1% OsO_4 in sodium phosphate or sodium cacodylate buffer pH 7.2. Following the publication by Maupin-Szamier and Pollard (1978) describing the effects of prolonged OsO_4 exposure on pure actin filament networks, post-fixation time was reduced to ½ hour. Fixation, wash and post-fixation was carried out at $4^{o}C$.

Rapid dehydration in a graded series of ethyl alcohols (70%, 80%, 95%, 100%) at $-20^{o}C$ followed post-fixation in OsO_4. Tissues remained in each change of alcohol a maximum of 30 seconds until reaching 100% ethyl alcohol. Then vials containing the tissues were allowed to warm to room temperature. The 100% ethyl alcohol was changed a minimum of 4 times, ½ hour each change. A mixture of 1 part 100% ethyl alcohol: 1 part propylene oxide replaced the final alcohol change and was followed by two changes of 100% propylene oxide, 15 minutes per change.

After propylene oxide, ovaries were placed in an infiltrating solution of Epon - Araldite (by volume, 2.5 parts Epon 812: 2 parts Araldite 502: 6 parts DDSA, Anderson and Ellis, 1965) containing between 2% and 4% DMP-30 plus an equal volume of propylene oxide. Infiltration proceeded for at least 24 hours