

ABSENCE OF RIBOSOMAL DNA AMPLIFICATION IN
THE MEROISTIC (TELOTROPHIC) OVARY OF THE
LARGE MILKWEED BUG *ONCOPELTUS FASCIATUS* (DALLAS)
(HEMIPTERA: LYGAEIDAE)

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ABSTRACT

In the typical meroistic insect ovary, the oocyte nucleus synthesizes little if any RNA. Nurse cells or trophocytes actively synthesize ribosomes which are transported to and accumulated by the oocyte. In the telotrophic ovary a morphological separation exists, the nurse cells being localized at the apical end of each ovariole and communicating with the oocytes via nutritive cords. In order to determine whether the genes coding for ribosomal RNA (rRNA) are amplified in the telotrophic ovary of the milkweed bug *Oncopeltus fasciatus*, the percentages of the genome coding for ribosomal RNA in somatic cells, spermatogenic cells, ovarian follicles, and nurse cells were compared. The oocytes and most of the nurse cells of *O. fasciatus* are uninucleolate. DNA hybridizing with ribosomal RNA is localized in a satellite DNA, the density of which is 1.712 g/cm^{-3} . The density of main-band DNA is 1.694 g/cm^{-3} . The ribosomal DNA satellite accounts for approximately 0.2% of the DNA in somatic and gametogenic tissues of both males and females. RNA-DNA hybridization analysis demonstrates that approximately 0.03% of the DNA in somatic tissues, testis, ovarian follicles, and isolated nurse cells hybridizes with ribosomal RNA. The fact that the percentage of DNA hybridizing with rRNA is the same in somatic and in male and female gametogenic tissues indicates that amplification of ribosomal DNA does not occur in nurse cells and that if it occurs in oocytes, it represents less than a 50-fold increase in ribosomal DNA. An increase in total genome DNA accounted by polyploidization appears to provide for increasing the amount of ribosomal DNA in the nurse cells.

Amplification of the DNA coding for ribosomal RNA (rDNA) occurs in the oocytes of a wide variety of organisms (Gall, 1969; Brown and Dawid, 1969). During the amplification process the genes coding for 18S and 28S ribosomal RNA (rRNA) and an associated "spacer" DNA are spe-

cifically replicated in the absence of replication of the remainder of the genome (Gall, 1968; Brown and Dawid, 1968; Dawid et al., 1970). The amplification process provides DNA template for the massive accumulation of ribosomes which occurs in the oocyte. These ribosomes serve as the protein

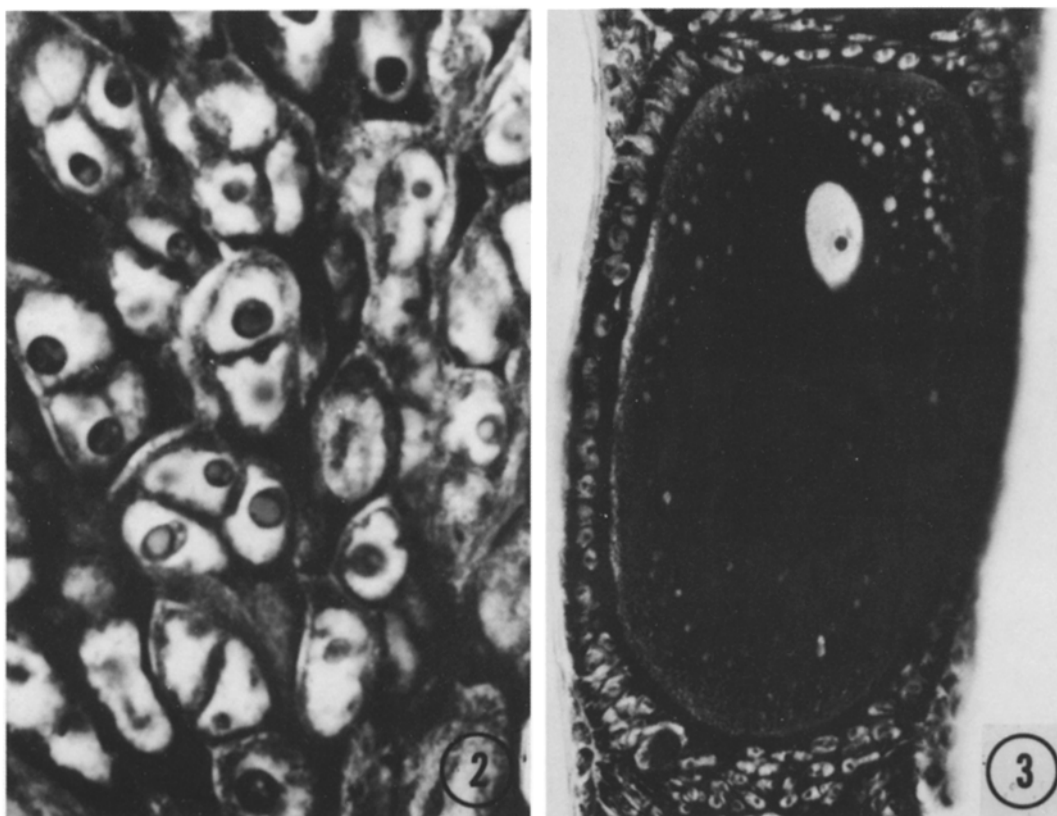
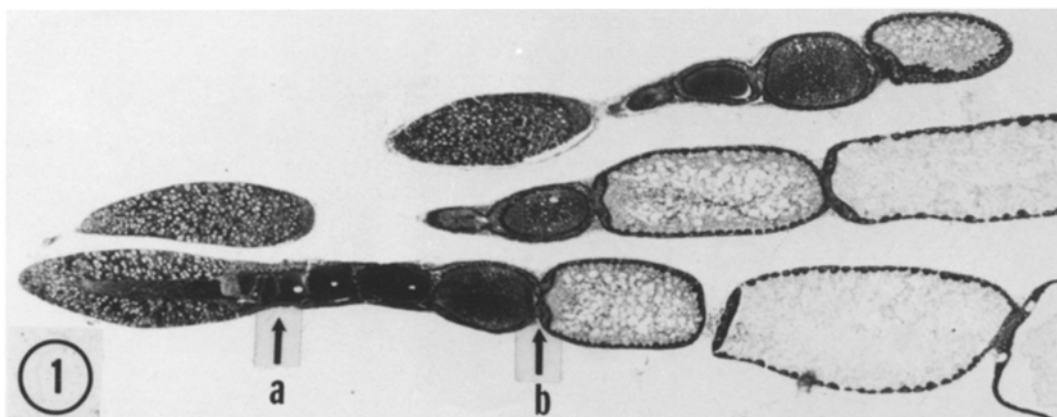


FIGURE 1 Azure B-stained section through several ovarioles in a postvitellogenic ovary of *O. fasciatus*. Ovariole tips composed principally of nurse cells were collected for DNA extraction by separation of the tips from the early oocytes at *a* (arrow). Previtellogenic oocytes were separated from postvitellogenic oocytes at *b* (arrow). DNA extracted from the region between *a* and *b* comprises that of previtellogenic oocytes and follicle cells. $\times 50$.

FIGURE 2 Azure B-stained section through an ovariole tip. Several large nurse cells are visualized. The nucleus of the nurse cells is distinguished by a large singular nucleolus. $\times 1,700$.

FIGURE 3 Azure B-stained section through a diplotene-stage oocyte. Vitellogenesis has begun in the cytoplasm. The nucleolus is a singular prominent structure within the germinal vesicle. $\times 500$.

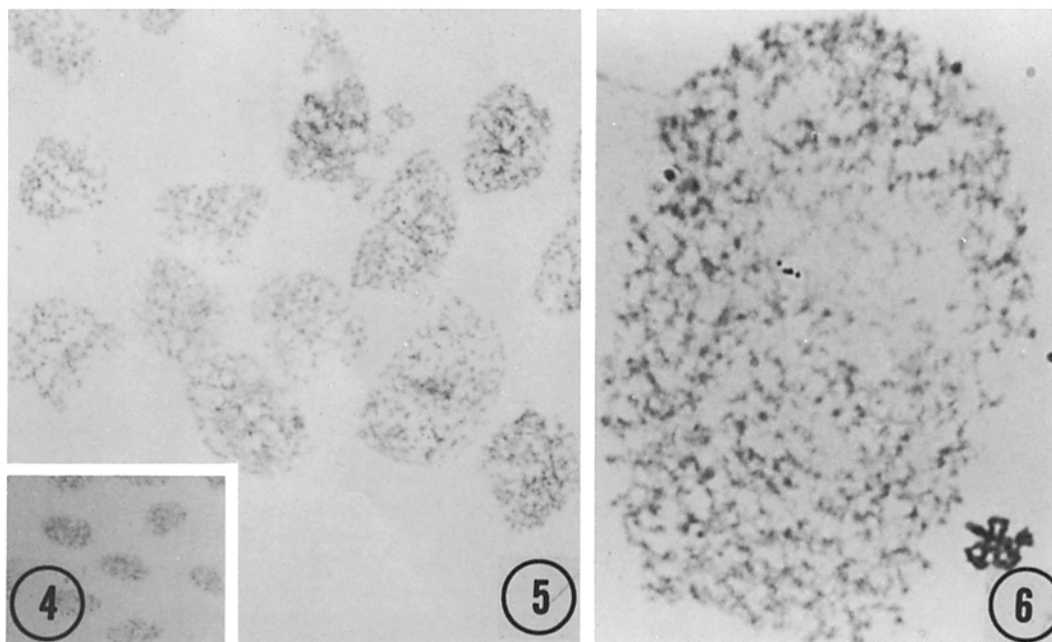
synthetic machinery of early embryogenesis (Brown and Littna 1964; Brown and Gurdon, 1964; Hansen-Delkeskamp, 1969).

The ovaries of insects demonstrate extensive variation in regard to mechanisms which contribute to oocyte development (see Bonhag, 1958, for review). Panoistic ovaries are characteristic of the older hemimetabolous orders of insects including the Thysanura, Odonata, Orthoptera, and Isoptera. Nutritive cells (trophocytes or nurse cells) are not found in the panoistic ovary. The oocyte nucleus synthesizes RNA, the chromosomes exhibiting a well-developed lampbrush-type appearance, and typically demonstrates hundreds of nucleoli which are actively synthesizing rRNA (Kunz, 1967). Amplification of rDNA is correlated with the formation of these multiple nucleoli and in many cases is associated with the appearance of large extrachromosomal DNA bodies about which the nucleoli form (Lima-de-Faria and Moses, 1967; Cave and Allen, 1969 *a*; Kato, 1968). rDNA amplification and the presence of such DNA bodies are not, however, limited to oocytes within panoistic ovaries (see Discussion).

Meroistic ovaries are characteristic of the mod-

ern orders of insects including the Hemiptera, Coleoptera, Lepidoptera, and Hymenoptera. Nurse cells or trophocytes are a characteristic feature of the meroistic ovary. In the typical meroistic ovary the germinal vesicle nucleus is quiescent in regard to RNA synthesis, the chromosomes being condensed into a small heteropyknotic karyosphere (Bier et al., 1967). The RNA synthetic activities of the oocyte appear to be taken over by the nurse cells, the nuclei of which are actively synthesizing RNA and demonstrate highly active nucleoli (Bier, 1967; Bier et al., 1967).

Two types of meroistic ovaries are distinguished. In the polytrophic ovary each oocyte is directly associated with a nest of 1–15 nurse cells, the nurse cells being daughter cells of the same cells that give rise to the oocyte (Koch et al., 1967). In an individual ovariole the nurse cells alternate with oocytes in a linearly sequential manner. In the telotrophic ovary all of the nurse cells serving the oocytes in an ovariole are clustered at the apical end of the ovariole. Communication of the nurse cells with the oocytes is accomplished by long cytoplasmic processes, nutritive cords, which extend from the trophocyte to the oocyte. The nurse



FIGURES 4–6 Feulgen-stained squash preparation of an ovariole tip. Few somatic cells can be visualized in the ovariole tip. The size of interphase somatic cell nuclei (Fig. 4) can be compared with the size of nurse cell nuclei (Fig. 5). The size of a large nurse cell nucleus can be compared with that of a mitotically dividing somatic cell (Fig. 6). Figs. 4 and 5, $\times 440$. Fig. 6, $\times 1,280$.

cells in the polytrophic and telotrophic ovary synthesize ribosomes which are transported to the oocyte (Hughes and Berry, 1970).

In order to determine whether the genes coding for rRNA are amplified in the typical telotrophic ovary, the proportion of the genome coding for rRNA in ovarian follicles and nurse cells of the large milkweed bug *Oncopeltus fasciatus* was compared with that of spermatogenic cells and somatic cells.

MATERIALS AND METHODS

Collection of Tissue

Eggs of *O. fasciatus* were accumulated over a 12-h period and incubated at 25°C until hatching (168 h). The nymphs which developed synchronously were reared in screen-covered pans and fed water and dried milkweed seeds. They received 14 h of light and 10 h of dark daily. Tissues were collected from 4–6-wk old adults (before vitellogenesis begins in the ovary), 9–12-wk old adults (after vitellogenesis has begun), and from prehatching embryos (144–156-h eggs). Collection was accomplished under a dissecting microscope. Ovariole tips were separated from early oocytes by microdissection of 70% ethanol-fixed postvitellogenic ovarioles. Tissues were stored at –70°C.

Preparation of DNA

DNA was extracted from homogenates of dechorionated embryos (Lagowski et al., 1973). The eggs were stored frozen at –70°C. The embryos (about 2 g) were homogenized in a motor-driven glass-Teflon homogenizer in 2 ml 0.1 M ethylenediaminetetraacetic acid (EDTA), 0.5% Sarkosyl NL-97 (Geigy Chemical Corp., Ardsley, N. Y.), and 0.05 M Tris, pH 8.5. The homogenate was then centrifuged at 1,900 g for 10 min and the supernate dialyzed against 0.02 M Tris, pH 8.5. DNA in the dialysate was banded in CsCl solution in the preparative ultracentrifuge as described below.

Testis, ovaries, ovariole tips, and ovarian follicles, which were stored in 70% ethanol, were washed with several changes of 2 × SSC. They were extracted with 100 µg/ml beta-amylase (Sigma Chemical Co., St. Louis, Mo.) for 1 h at 37°C, and then with 100 µg/ml RNase-A (Worthington Biochemical Corp., Freehold, N.J.) for 1 h at 37°C, and subsequently with 1 mg/ml Pronase (Calbiochem, San Diego, Calif.) for 2 h at 37°C. The digest was centrifuged at 1,900 g and the supernate dialyzed against 0.02 M Tris, pH 8.5.

DNA was routinely banded by preparative ultracentrifugation in CsCl. The dialysates containing 0.1–1.0 mg DNA were diluted to 5 ml in CsCl solution which was brought to density 1.70 g/cm³ and centrifuged for 60 h, 20°C, 120,000 g in the Spinco SW-50.1 rotor (Beckman Instruments, Spinco Div., Palo Alto, Calif.). Individual

fractions were collected and the DNA-containing fractions identified by UV spectrophotometry. Fractions containing DNA were pooled and dialyzed overnight against 0.1 × SSC.

The purified DNA was characterized by isopycnic banding in CsCl in a Beckman Model E ultracentrifuge which was equipped with a monochromometer and multiplex scanning device (Beckman Instruments).

Preparation of Labeled RNA

Difficulties in preparing labeled RNA of sufficient specific activity for hybridization experiments precluded the use of *O. fasciatus* rRNA. Previous studies have demonstrated extensive homologies between the rRNAs of a wide variety of organisms (Sinclair and Brown, 1971). The rRNA of the South African clawed toad *Xenopus laevis* has been utilized extensively in such

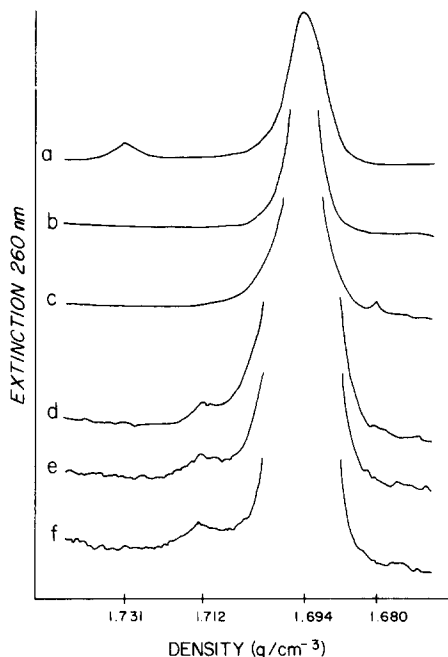


FIGURE 7 Buoyant density profiles of various *O. fasciatus* DNA preparations determined in neutral CsCl. (a) 0.2 A_{260} U of DNA from testis with *Micrococcus lysodeikticus* DNA (0.04 A_{260} U, $\rho = 1.731$ g/cm³) as an internal density marker. (b, c) 0.2 A_{260} U of DNA from (b) previtellogenic and (c) postvitellogenic ovaries. (d–f) 0.6 A_{260} U of DNA from (d) 132–144-h embryos (embryo DNA), (e) ovariole tips (nurse cell DNA), and (f) previtellogenic oocytes and follicle cells. The small peaks at density 1.712 in d, e, and f represent 0.2% of the DNA. Samples were centrifuged at 40,000 rpm for 18 h at 20°C with 2° double-sector centerpieces in the An-GTi rotor of the Beckman Model E ultracentrifuge. Scans were made at a wavelength of 260 nm.

hybridization experiments. Labeled 18S and 28S RNA were prepared by growing a cell line of *X. laevis* kidney cells (line CCL 102A obtained from the American Type Culture Collection, Rockville, Md.) in modified Leibovitz L-15 medium with 15% fetal calf serum. Cells were grown for 3–5 days in medium containing 25 $\mu\text{Ci/ml}$ [$5\text{-}^3\text{H}$]uridine (sp act 29.2 Ci/mmol, New England Nuclear, Boston, Mass.) and then for an additional 24 h in two changes of fresh medium containing unlabeled uridine. Subsequently, RNA was extracted from the cells by a modification of the phenol procedure and 18S and 28S RNA were isolated as described elsewhere (Cave, 1972). Ribosomal RNA was reconstituted by combining one part 18S RNA with two parts 28S RNA (wt/wt).

Nucleic Acid Hybridization

RNA-DNA hybridization analysis was carried out on Millipore HAWP filters (Millipore Corp., Bedford, Mass.) as described previously (Cave, 1972).

RESULTS

The ovaries of *O. fasciatus* are composed of eight separate ovarioles. The nurse cells are localized at the anterior end of each ovariole. Microdissection of ovariole tips taken from ovaries after vitellogenesis has begun enables the preparation of nurse cell DNA (Fig. 1). DNA from the adjacent four to five previtellogenic oocytes comprises oocyte and follicle cell DNA (ovarian follicle DNA) in the absence of nurse cell DNA (Fig. 1). A single prominent nucleolus is visualized within the germinal vesicle of *O. fasciatus*. The nucleolus remains singular long after vitellogenesis has begun (Fig. 3). Like the oocyte nuclei, most of the nurse cell nuclei contain but a single nucleolus (Fig. 2). Cell counts made over sections of 10 different ovariole tips show that more than 95% of the cells in the ovariole tip are nurse cells. The remainder of the cells are sheath cells, follicle cells, or young oocytes. The nurse cell nuclei are markedly larger than somatic cell nuclei (Figs. 4–6). Three zones of trophocyte development are visualized in the ovariole tip (Bonhag, 1955). Proceeding from zone I to zone III, the nurse cell nucleus gradually increases in size. About 70% of the cells in the ovariole tip are large zone III nurse cells, the average nuclear volume of which is $302 \pm 23.1 \mu\text{m}^3$ (about 10 times the volume of a somatic cell nucleus). Microphotometric measurements made on trophocyte nuclei and somatic cell nuclei of Feulgen-stained, squashed ovariole tips indicate that 70% of the trophocyte nuclei have DNA contents in the 32C–64C classes (E. Rasch, per-

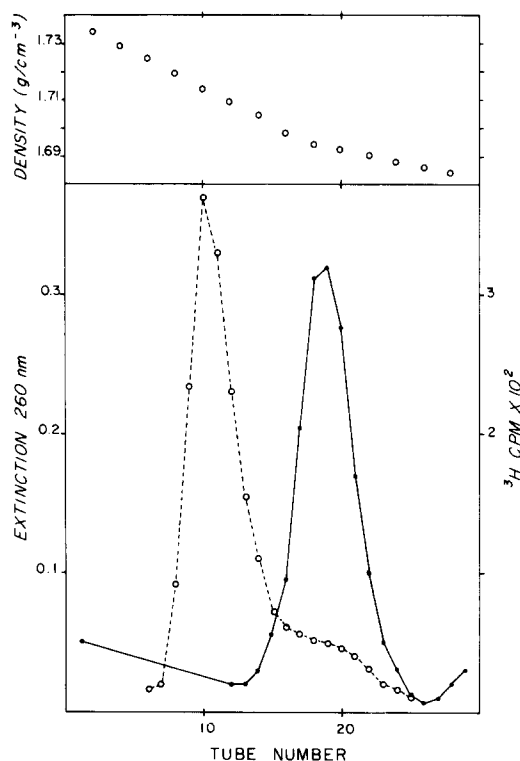


FIGURE 8 Buoyant density of DNA hybridizing with rRNA. DNA from the ovaries of female *O. fasciatus* was sedimented to equilibrium in neutral CsCl and the gradients were fractionated. The density of each fraction was determined from the relationship between density and refractive index (Ifft, 1961). The fractions were diluted to 1 ml with distilled water and the optical density (260 nm) was determined. The DNA in each fraction was denatured and bound to filters. The filters were hybridized in a solution containing 1 $\mu\text{g/ml}$ *X. laevis* rRNA (sp act 265,000 cpm/ μg). Circles joined by solid line, optical density 260 nm; open circles joined by dotted line, radioactivity on filters; open circles unjoined, buoyant density.

sonal communication). Nurse cell nuclei containing up to 256 times the haploid amount of DNA were the largest measured.

DNA extracted from various tissues of *O. fasciatus* and centrifuged to equilibrium in CsCl in the analytical ultracentrifuge forms two bands. The main band has a buoyant density of 1.694 gm/cm^3 (Fig. 7 a). A minor satellite band with a buoyant density of 1.712 gm/cm^3 is visualized when the gradients are overloaded with DNA (Fig. 7 d–f). The satellite accounts for approximately 0.2–0.3% of the DNA in all of the tissues that were

examined. A second satellite DNA with a density of 1.680 is found in DNA extracted from postvitellogenic ovaries (Fig. 7 c). This satellite is not detectable in other tissues and is resistant to further amylase and RNase digestion. It may represent mitochondrial DNA which has accumulated in the eggs. The calculated percent guanine and cytosine (GC) ratios of the main band, minor satellite, and light satellite (found only in postvitellogenic ovaries) are 33–34% GC, 53% GC, and 25% GC, respectively. Hybridization of DNA centrifuged to equilibrium on preparative CsCl gradients with labeled rRNA indicates that DNA hybridizing with rRNA is of the same density ($\rho = 1.712$) as the minor satellite DNA which was visualized in the analytical ultracentrifuge (Fig. 8). The data indicate that DNA extracted from somatic tissues, testis, ovarian follicles, and nurse

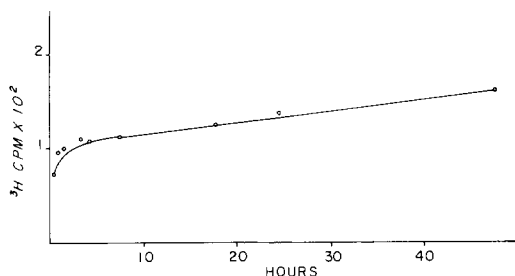


FIGURE 9 Filters containing DNA from the testis of *O. fasciatus* were hybridized with ^3H -labeled *X. laevis* rRNA (sp act 265,000 cpm/ μg). Each filter contained 2.5 μg unfractionated DNA. Filters were hybridized in solution containing 2 $\mu\text{g}/\text{ml}$ rRNA in $4 \times \text{SSC}$ -0.4% SDS at 68°C .

cells contains the same proportions of ribosomal satellite DNA and main-band DNA.

DNA extracted from various tissues of *O. fasciatus* was denatured, bound to filters, and hybridized with labeled rRNA. The reaction approaches saturation in 3–4 h under the hybridization conditions utilized (Fig. 9). When hybridized with increasing concentrations of rRNA, DNA extracted from 132–144-h whole embryos, testis, previtellogenic ovaries, postvitellogenic ovaries, ovarian follicles, and isolated nurse cells demonstrates similar hybridization kinetics (Fig. 10). The fact that the percent of DNA hybridizing with rRNA is the same for ovariole tips, testis, and somatic tissues indicates that amplification of rDNA does not occur in nurse cells of *O. fasciatus*.

Ovarian follicle DNA hybridizes with rRNA to the same extent as does DNA from testis and somatic tissues. Cell counts made on Feulgen-stained squash preparations of ovarian follicles (between arrows *a* and *b*, Fig. 1) demonstrate that there are 52.06 ± 5.7 follicle cell nuclei per oocyte nucleus. Due to the presence of follicle cell DNA, amplification of oocyte rDNA to a level less than 50 times the testis-somatic cell level cannot be ruled out. If amplification does occur in oocytes of *O. fasciatus* it represents less than a 50-fold increase in rDNA.

Approximately 0.033% of the DNA of *O. fasciatus* hybridizes with *X. laevis* rRNA (Fig. 11). On the basis of renaturation kinetics, the haploid genome size for *O. fasciatus* is $2.8\text{--}3.7 \times 10^{12}$ daltons (Lagowski et al., 1973). Assuming that the

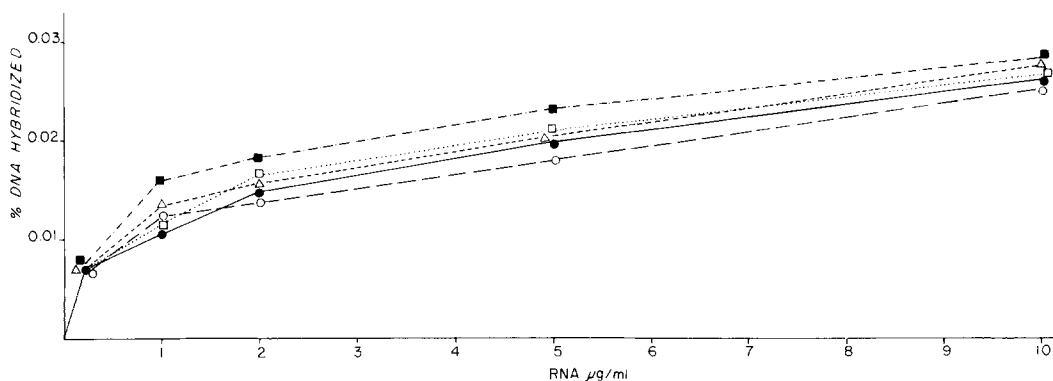


FIGURE 10 Filters containing DNA extracted from whole embryos (\square), ovaries (\circ), testis (\blacksquare), ovarian follicles (\triangle), and nurse cells (\blacksquare) were hybridized with ^3H -labeled *X. laevis* rRNA (sp act 265,000 cpm). Filters were hybridized for 18 h under the same conditions as those described in Fig. 6. The amount of DNA on each filter was determined after hybridization by hydrolyzing the filters in 5% perchloric acid for 30 min at 70°C and measuring the A_{260} of the hydrolysate.

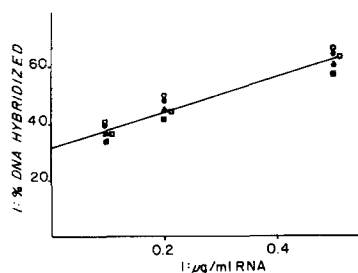


FIGURE 11 Double reciprocal plot of the data in Fig. 10. The symbols are the same as those used in Fig. 10.

molecular weight of *O. fasciatus* rRNA is approximately the same as that of *X. laevis* 18S and 28S RNA (2.2×10^6 daltons) (Dawid et al., 1970), there are estimated to be 210–270 copies of the genes coding for rRNA per haploid genome. This is a minimum estimate since the percent of homology between the rRNA of *X. laevis* and that of *O. fasciatus* has not been determined.

DISCUSSION

In insects, amplification of the genes coding for rRNA has been correlated in many cases with the presence of large extrachromosomal DNA bodies within the oocyte nucleus. In the panoistic ovary of the house cricket *Acheta domesticus* (Orthoptera), such a DNA body is visualized in pachytene and early diplotene stage oocytes (Nilsson, 1966; Cave and Allen, 1969 a; Kunz, 1969). The oocyte nucleus contains approximately 100 times as much DNA hybridizing with rRNA as does a somatic cell nucleus, yet the total amount of DNA in the oocyte nucleus is approximately equivalent to the 4C somatic cell amount of DNA (Lima-de-Faria et al., 1969; Cave and Allen, 1969 b; Cave, 1972). *In situ* hybridization with rRNA demonstrates that the amplified rRNA is localized in the DNA body (Cave, 1972; Cave, 1973; Ullman et al., 1973).

Similar DNA bodies have been described in the meroistic (polytrophic) ovaries of several representatives of the Dytiscidae (Coleoptera) and Tipulidae (Diptera) (Giardina, 1901; Urbani, 1969; Bayreuther, 1952). Amplification of the genes coding for rRNA has been correlated with the presence of such DNA bodies in the Dytiscidae (Gall et al., 1969). Unlike the oocytes in typical meroistic ovaries, the oocytes of these Dytiscidae and Tipulidae develop active nucleoli during the diplotene stage of meiosis (Urbani and Russo-Caia, 1964; Kato, 1968; Lima-de-Faria and Moses, 1967).

Nurse cells are derived from the same cells that give rise to the oocyte (Koch et al., 1967). It is unclear whether nurse cells also amplify their rDNA. In the polytrophic ovary of *Tipula oleracea* a DNA body is also present in some nurse cell nuclei, where it is associated with the nucleolus (Lima-de-Faria and Moses, 1966). Large numbers of nucleoli develop within the nurse cell nuclei of *Calliphora erythrocephalus*. In specific strains of *C. erythrocephalus*, which show polytenization of nurse cell chromosomes, the nucleoli are seen to be free of the chromosomes, in contrast to other polytene tissues where the nucleolus is attached to a specific chromosomal locus (Ribbert and Bier, 1969). The free nucleoli, in which DNA particles can be visualized by histochemical techniques, are known to incorporate [³H]uridine. These data suggest that in addition to polyploidization, the nurse cells may amplify their nucleolar organizer DNA (rDNA).

Previous studies suggest amplification of rDNA in meroistic ovaries in which extrachromosomal DNA bodies have not been detected. DNA extracted from the meroistic ovary of *Rhynchosciara angelae* (Diptera) contains twice as much DNA hybridizing with rRNA as does DNA extracted from salivary glands (Gambarini and Meneghini, 1972). Since DNA complementary to rRNA is underreplicated in salivary glands of other Dipterans (Hennig and Meer, 1971), it is not clear whether differences in the amount of rDNA reflect amplification of the somatic cell value in the ovary, or underreplication in salivary gland cells. In contrast to these data, the percent of DNA hybridizing with rRNA in the polytrophic ovary of *Drosophila melanogaster* (Diptera) is the same as that of the total DNA extracted from adult flies (Mohan and Ritossa, 1970).

In the typical meroistic ovary the chromosomes condense to form a karyosphere and do not synthesize RNA. Nucleoli, if present, do not synthesize RNA. The ovaries of *O. fasciatus* are exceptional in that the germinal vesicle synthesizes nucleolar and chromosomal RNA (Zinneister and Davenport, 1971). However, most of the RNA which accumulates in the oocyte is synthesized by the nurse cells and is communicated to the oocytes or nurse cells of *O. fasciatus*.

Nuclei of nurse cells in the telotrophic ovary of *Acanthocephala bicoloripes* (Hemiptera) show a gradual increase in nuclear diameter as one proceeds from the anterior toward the posterior end of the ovariole tip. These nuclei contain up to 70

times the haploid amount of DNA (Schrader and Leuchtenberger, 1951). A correlation between nuclear size and DNA content was noted.

The nurse cells of *O. fasciatus* become highly polyploid as a result of cell fusion (Bonhag, 1955). Most of the nurse cell nuclei contain 32–64 times the haploid amount of DNA, their volume being about 10 times the volume of a somatic cell nucleus. A single nucleolus is found in each nurse cell nucleus. The present data demonstrate that the nurse cells contain the same proportion of ribosomal genes as do somatic cells. Polyploidization of the entire chromosome complement appears to increase the amount of rDNA in the nurse cells, providing increased DNA template for the massive accumulation of rRNA which occurs in the oocyte.

Amplification of rDNA has been correlated with the number of nucleoli present in an oocyte. In the uninucleolate oocyte of the starfish, amplification of rDNA was not detected, while it was readily observed in the multinucleolate oocyte of the striped bass (Vincent et al., 1969). More recent studies on the uninucleolate oocyte of the marine worm *Urechis caupo* demonstrate six- to eightfold amplification of the genes coding for rRNA (Dawid and Brown, 1970). The oocytes and nurse cells of *O. fasciatus* contain a singular nucleolus. The nucleolus remains singular in the nurse cells in spite of extensive polyploidization. If amplification of rDNA does occur in oocytes of *O. fasciatus* it represents less than a 50-fold increase in rDNA.

The results of the present investigation are in agreement with previous physical measurements made on DNA of *O. fasciatus*. On the basis of the GC content of rRNA, Lagowski et al., (1973) proposed that a high density satellite DNA ($\rho = 1.712$) contained the genes which code for rRNA. Physical determinations of buoyant density, thermal stability, and base ratio demonstrated that the main band DNA had a density of 1.6937 in CsCl and contained 32–34% GC. The satellite DNA ($\rho = 1.712$) accounts for about 0.2% of the total DNA. RNA-DNA hybridization analysis confirms that the satellite DNA does indeed contain sequences which are complementary to rRNA.

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