RNA SYNTHESIS AND HETEROCHROMATIZATION IN EARLY DEVELOPMENT OF A MEALYBUG[1]

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ABSTRACT

Microinjection of specific radioisotope tracers showed that DNA and protein synthesis are triggered by fertilization but nuclear RNA synthesis does not occur until the cleavage nuclei migrate to the periphery. In the male, heterochromatization of the paternal chromosome set also occurs when the cleavage nuclei reach the periphery. These results help to explain those of several types of genetic test, especially the failure of typical dominant lethal induction in the heterochromatized set.

IN the mealybug, the paternal set of chromosomes becomes heterochromatic during the early embryogeny of the male and remains so in many tissues, including the germ line, during subsequent development; the maternal chromosome set remains euchromatic throughout. Genetic inactivity of the paternal set has been demonstrated in several ways. Whether mutant or wild type, only the maternal genes are expressed in the sons (BROWN and NUR 1964; BROWN and WIEGMANN 1969). The paternal, heterochromatic set fails to make ribonucleic acid (RNA) as measured by tritiated uridine labeling while the maternal, euchromatic set in the same nuclei incorporates the label (BERLOWITZ 1965; SABOUR 1969). Paternal treatment with X rays or radiocobalt at doses up to 30,000r or rep fails to induce dominant lethality in the sons (BROWN and NELSON-REES 1961), but those tissues in which the heterochromatization of the paternal set is reversed during embryogeny show derangements analogous to dominant lethal effects (NUR 1967). The survival of all the sons after fairly high doses of ionizing irradiation is presumptive evidence that the paternal genes are inactive during the 5 or 6 cleavages prior to the onset of heterochromatization. It will be shown in this report that the onset of gene action, as indicated by the synthesis of RNA in nuclei, is closely correlated, in respect to both time and place, with the onset of heterochromatization.

MATERIALS AND METHODS

Because of its relatively large cells and clear cytology, the mealybug, *Pseudococcus obscurus* Essig, was used as the experimental subject. In mealybugs, fertilization occurs *in situ* in the

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ovariole, and early stages of embryonic development take place before the eggs are deposited. Females produce 200 to 500 eggs over a period of about 10 days and there is thus no synchrony in embryonic development. Most of the morphogenesis of the embryo occurs inside the mother but the individual eggs are protected by an impermeable covering formed a few hours before fertilization. The nature of mealybug embryogeny makes the application of ordinary biochemical methods impractical. Eggs were taken from the mother and placed on microscope slides coated with a thin gelatin layer (prepared with a 12% w/v aqueous gelatin solution) which held the eggs firmly for microinjection. Micropipettes were made with a tip of 5–7μ outside diameter and calibrated according to the method of LIN (1966). With the aid of a Lietz micromanipulator volumes of 0.02 ± 0.005 nl of one of the following labeling compounds was injected: 1 mc/ml thymidine methyl-^{3}H, specific activity, 6.4 c/mM (Schwartz Bioresearch, Inc.): 1 mc/ml uridine-5-^{3}H, specific activity, 25.5 c/mM (Nuclear Chicago); 0.5 mc/ml L-leucine-5-^{3}H, specific activity, 23.5 c/mM (Nuclear Chicago). For control injections, insect Ringer's solution was used without isotope tracers. Egg membranes of embryonic states under study did not resist the micropipette and, with the volumes of solution injected, there was no leakage of material out of the egg. The early stages of embryonic development can be gauged only after staining so that many had to be injected to obtain a few at the critical stages. After injection, the eggs were incubated in a moist chamber at room temperature, 22.0 ± 0.5°C. An incubation period of 30 min for ^{3}H-thymidine and ^{3}H-leucine produced good results. For quantitative grain analysis it was found that 2 to 4 hr of ^{3}H-uridine incorporation (for the exposure time described below) produced the best results. However, due to absence or very low incorporation of ^{3}H-uridine by the earlier cleavage stages in some experiments, incubation time for these was extended to 6 hr in some trials. After incubation, the eggs were fixed for 2 hr in Bradley-Carnoy fixative (BRADLEY 1948) and then either sectioned at 4–6μ or squashed in 45% acetic acid. Identification of compounds in labeled cells was corroborated by enzymatic hydrolysis of alternate sections and of some squashes with trypsin (2× crystallized, Worthington Biochemical Co.), 0.2 mg/ml in 0.05M Tris buffer containing 0.01M $CaCl_2$ at pH 7.6 for 20 min at 37°C; ribonuclease A (RNase) (5× crystallized, protease-free, bovine pancreatic, Sigma Chemical Co.), 0.2 mg/ml in 0.05M Veronal-acetate buffer at pH 6.6 for 2, 4 or 6 hr at 37°C; deoxyribonuclease I (DNase) (bovine pancreatic, RNase-free, Worthington Biochemical Co.), 0.2 mg/ml in 0.05M Veronal-acetate buffer at pH 6.6 containing 0.003M $MgSO_4$, for 4 hr at 37°C; control slides were treated with the same buffers and at the same temperature but without enzymes. The slides were placed in 5% trichloroacetic acid (TCA) at 4°C for 10 min, washed thoroughly in water, and air dried. For autoradiography, slides were coated with Ilford K.5 liquid emulsion, diluted 1:1 with distilled water, treated in a hydrogen peroxide (H_2O_2) chamber for 2 hr (CARO 1964) to eradicate background silver grains, and stored at 4°C in light-tight boxes containing Drierite. After an exposure of 11 days the slides were developed in Kodak D-19 and were stained with azure B (FLAX and HIMES 1952) or Harris' hematoxylin. In some RNA labeling experiments, an exposure time of 2 months was used before development. All young embryos, through mid-cleavage, and a random selection of older embryos were used for grain counts. In embryos of 10 nuclei or less, all were used; if more, either 10 or 20 nuclei were randomly selected. Autoradiographs of sections were used to determine the stage and place where label appeared.

RESULTS AND DISCUSSION

Embryogenesis in *P. obscurus* follows the pattern known for other mealybugs (SHINJI 1919; SCHRADER 1923a and b). Divisions are not completely synchronous but sufficiently so that conventional designations may be used. The first five cleavages occur in the central part of the egg. Heterochromatization begins between the 5th and 6th cleavage, shortly after migration of nuclei to the periphery in those embryos destined to become males (NUR 1967; SABOUR 1969). Very early embryogeny can thus be conveniently divided into early cleavage (2–32

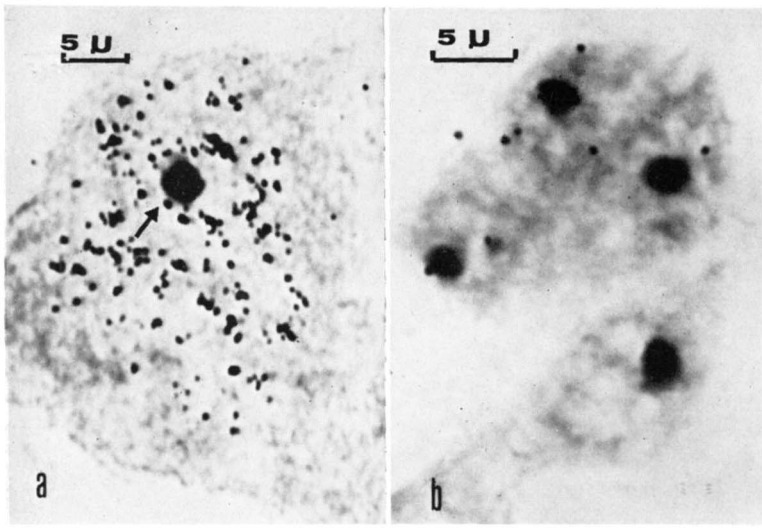

FIGURE 1.—Nuclear labeling after injection of *P. obscurus* male blastoderm embryos with ^{3}H-thymidine; incubated 3.5 hr; squash preparation; 12 days exposure. a, Labeled nucleus; grains lacking over heterochromatic set (arrow); azure B stain. b, Control; 4 hr DNase; most label removed; Harris' hematoxylin.

nuclei), mid-cleavage (33–128 nuclei), and late cleavage (129–300 nuclei). Cell walls are formed around the nuclei at the blastoderm stage (300–500 nuclei); a germ band next develops and produces an S-shaped embryo extending the length of the egg.

Autoradiography indicated that syntheses of both deoxyribonucleic acid (DNA) and protein begin with fertilization and are thereafter maintained. These results thus serve as a control for the RNA experiments. No label above background was observed in unfertilized eggs even after incubation periods up to 7 hr with either tritiated thymidine or leucine followed by long exposures before development.

After an incubation of 30 minutes with ^{3}H-thymidine, label was specifically located over the nuclei of all stages of early embryogeny (Figure 1a), and longer incubation gave correspondingly denser labeling. Four hours of DNase hydrolysis of either squashes or sections almost completely eliminated the nuclear labeling (Figure 1b): these results suggested that the injected ^{3}H-thymidine was specifically incorporated into high molecular weight DNA. Treatment with buffer solution, ribonuclease, hot 1N HCl (10 min at 60°C), or cold 5% TCA did not remove the nuclear labeling.

^{3}H-leucine was incorporated into the protein of all stages of early embryogeny with about equal intensity. An incubation of 30 min to one hour was sufficient to produce heavy labeling. Trypsin hydrolysis of paraffin sections for 20 min reduced the grain density considerably.

In early cleavage stages, ^{3}H-leucine incorporation was located over patches of basophilic protoplasm distributed throughout the embryo. As cleavage progressed,

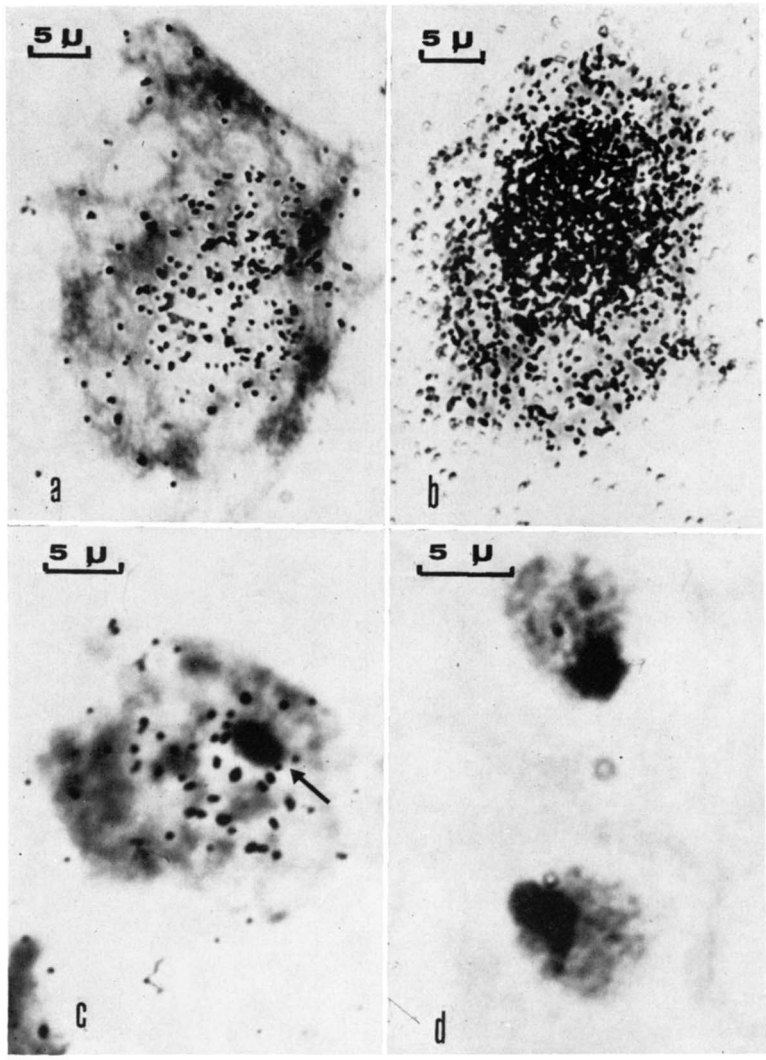

FIGURE 2.—Nuclear labeling after injection of *P. obscurus* embryos with ^{3}H-uridine; squash preparations; 11 days exposure; Harris' hematoxylin. a, Female, mid-cleavage (*ca.* 100 nuclei); incubated 2 hr b, Female, late cleavage (*ca.* 160 nuclei); incubated 4 hr c, Male, blastoderm (*ca.* 350 nuclei); label over eu- but not heterochromatin (arrow). Control; 4 hr incubation; 2.5 hr RNase.

the basophilic material became closely associated with the nuclei and the label continued to be concentrated over this material both before and just after migration of the nuclei to the periphery.

As measured by autoradiography, RNA synthesis varied considerably in accord with developmental stage (Table 1). Regardless of stage or location in the embryo, the ^{3}H-uridine label was removed completely by a 2-hr exposure to RNase (Figure 2d), but was unaffected by the enzyme-free buffer under similar

TABLE 1

Averages of numbers of grains per nucleus after injection of different embryonic stages of Pseudococcus obscurus *with* ^{3}*H-uridine**

	Eggs and embryos		Grain counts	
	Number analyzed	Total number nuclei present	Number nuclei analyzed**	Grains per nucleus av. ± S.E.
Mature meiotic egg	36	1	1	0
Cleavage:				
Early (1–32 nuclei)	13	4–32	4–10	0†
Mid (33–128 nuclei)‡	2	42, 47	20	8 ± 1.3§
	3	69, 70, 73	20	40 ± 3.4
Late (129–300 nuclei)	4	140–250	10	105 ± 3.8
Blastoderm	4	350–500	10	128 ± 5.6
Germ-band	5	—	10	17 ± 1.4
S-shaped embryo	6	—	10	12 ± 0.06

* Incubation with tracer, 4 hr; exposure, 11 days.

** Nuclei chosen at random, to 10 in early cleavage and later, and to 20 at the critical mid-cleavage stage.

† Grains were observed over dense basophilic protoplasmic areas adjacent to or surrounding nuclei; see text for further explanation.

§ Only 25% of nuclei showed 10 or more grains.

‡ Male embryos only from the time they were first identifiable during mid-cleavage.

conditions, observations which suggest that the injected ^{3}H-uridine is incorporated specifically into DNA-directed RNA molecules.

Embryos in early cleavage (2–32 nuclei), before nuclear migration to the periphery, showed no labeling when incubated for 2 hr with ^{3}H-uridine. However, after an incubation period of 3.5 hr or longer, grains were detected over the same basophilic areas which give heavy labeling with leucine. Only after incubation periods of 4 to 7 hr and exposure of one month were there some grains above background over cleavage nuclei, but this amounted to less than 10% of the total label. During early cleavage, the contents of the interphase nuclei were diffuse; there was no condensation of chromatin which might be held responsible for failure of these nuclei to be labeled. Trials of longer exposure periods, up to one month, correspondingly increased the average grain number per nucleus of later stages to the point where accurate grain counting would have been very difficult, but the results for unfertilized eggs and early cleavage remained the same.

Although these observations parallel those of the sea urchin embryo in which most ^{3}H-uridine incorporations during early cleavage are cytoplasmic and represent end-labeling of the transfer RNA (Malkin, Gross and Romanoff 1964; Gross, Kraemer and Malkin 1965), other types of cytoplasmic RNA labeling (Kalf 1964; Roodyn and Wilkie 1968) cannot be ruled out at present for the mealybug.

Sections of embryos showed that ^{3}H-uridine incorporation into cleavage nuclei begins with their migration to the egg periphery, but prior to appearance of the nucleolus (Figure 2a). The number of nuclei per embryo indicated that this

event occurs at the 5th to 6th cleavages. During this stage, only those nuclei that were located in the egg periphery were labeled, and thereafter longer incubation with ^{3}H-uridine increased the nuclear incorporation of the tracer accordingly (Figure 2b).

Nuclear RNA synthesis begins in embryos of both sexes when the nuclei first appear at the periphery of the embryo. In the male embryos, heterochromatization also begins in those nuclei which have reached the periphery. Although most of the nuclei at the periphery of male embryos show both RNA labeling and heterochromatin (Figure 2c), a few will have only the label or only the heterochromatin, or, very rarely, neither. These results indicate that the two processes, onset of gene action and heterochromatization, are probably not directly related to each other but are correlated through response to other developmental processes. The results also indicate that there is only a small percentage of nuclei at the periphery of these early male embryos in which transcription of the paternal set could be, but not necessarily is occurring. The absence of specific nuclear RNA synthesis prior to the 5th to 6th cleavages is in agreement with earlier work with the mealybug showing that dominant lethal effects induced by paternal treatment are expressed only in the daughters, not in the sons. However, since information regarding the pool size of RNA precursor in mealybug eggs is not available, and despite the high specific activity of ^{3}H-uridine used (25.5 c/mM) and long incubation period, RNA synthesis at a very low rate cannot be ruled out.

During late cleavage and blastoderm stages, the level of ^{3}H-uridine incorporation is increased for the same incubation period. This increase corresponds with the time of nucleolus appearance during which silver grains are clustered over nucleoli (Figure 3a, b). The nucleolus has been implicated in ribosomal RNA synthesis in both insects (RITOSSA and SPIEGELMAN 1965; RITOSSA, ATWOOD and SPIEGELMAN 1966) and vertebrates (PERRY 1967; BROWN and DAVID 1968). On this basis, the nucleolus of the mealybug is also probably engaged in rRNA synthesis, but there is no independent evidence since uridine labeling does not discriminate among classes of RNA. The peak of RNA synthesis occurs from late cleavage through blastoderm formation; the germ-band and S-shaped embryos show much lower uptake of ^{3}H-uridine per nucleus (Table 1). Female embryos follow the same pattern except that the rate of RNA synthesis per nucleus is about twice that per nucleus in a male embryo (SABOUR 1969).

To recapitulate, RNA synthesis in the mealybug begins when the nuclei migrate to the periphery at the 5th to 6th cleavages, increases markedly about the time of the appearance of the nucleolus at the 8th to 9th cleavages, and then decreases to quite a low level. These results parallel those observed in other insects. In certain coleopterans, RNA synthesis begins in the blastoderm stage after migration of the nuclei to the periphery (LOCKSHIN 1966). In the heteropteran, *Oncopeltus fasciatus*, ribosomal RNA synthesis is turned on at gastrulation but no nuclear RNA synthesis was observed at the earlier blastoderm stage (HARRIS and FORREST 1967).

Protein synthesis in *P. obscurus* embryos, on the other hand, begins following fertilization and continues thereafter at a rapid rate. It appears therefore, that

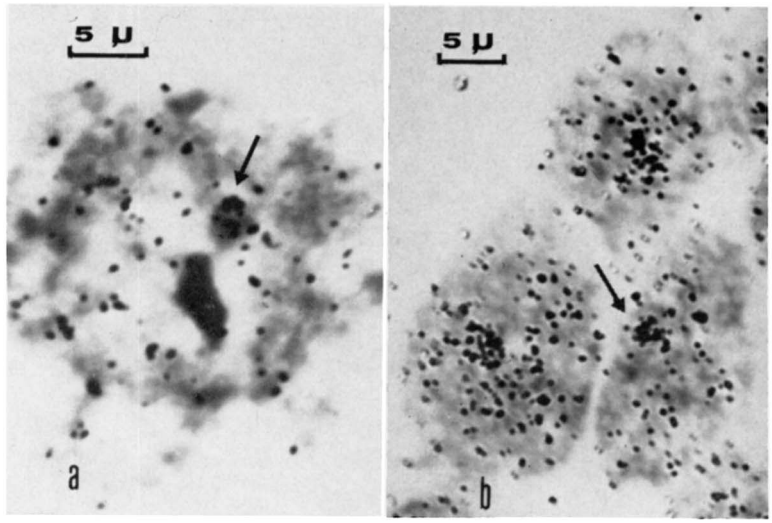

FIGURE 3.—Silver grain clusters over nucleoli (arrows) after ^{3}H-uridine injection of blastoderm embryos, *P. obscurus*; squash preparation; Harris' hematoxylin. a, Labeled nucleus from male (*ca.* 350 nuclei); incubated 2 hr b, Labeled nuclei from female (*ca.* 500 nuclei); incubated 4 hr.

fertilization activates the translation of pre-existing messenger RNA (mRNA). The existence of control is possible at the translational level, similar to that in other organisms (GROSS 1968) in which the utilization of maternal mRNA made earlier during vitellogenesis is triggered by fertilization.

The parallel between heterochromatization and the synthesis of RNA again suggests the importance of the molecular content of the egg periphery, formed during vitellogenesis, in initiating specific responses in early development (KRAUSE and SANDER 1962; DAVIDSON 1968).

LITERATURE CITED

BERLOWITZ, L., 1965 Correlation of genetic activity, heterochromatization, and RNA metabolism. Proc. Natl. Acad. Sci. U.S. **53:** 68–73.

BRADLEY, M. V., 1948 An acetocarmine squash technique for mature embryo sacs. Stain Technol. **23:** 29–40.

BROWN, D. D. and I. B. DAVID, 1968 Specific gene amplification in oocytes. Science **160:** 272–280.

BROWN, S. W. and U. NUR, 1964 Heterochromatic chromosomes in coccids. Science **145:** 130–136.

BROWN, S. W. and W. A. NELSON-REES, 1961 Radiation analysis of a lecanoid genetic system. Genetics **46:** 983–1007.

BROWN, S. W. and L. I. WIEGMANN, 1969 Cytogenetics of the mealybug *Planococcus citri* (Risso) (Homoptera:Coccoidea): genetic markers, lethals, and chromosome rearrangements. Chromosoma **28:** 255–279.

CARO, L. G., 1964 High-resolution autoradiography. Pp. 327–363. In: *Methods in Cell Physiology* Vol. 1. Edited by D. M. PRESCOTT.

DAVIDSON, E. H., 1968 *Gene Activity in Early Development.* Academic Press, New York. Pp. 103–164.

FLAX, M. H. and M. H. HIMES, 1952 A microspectrophotometric analysis of metachromatic staining of nucleic acids in tissues. Physiol. Zool. **25:** 297–311.

GROSS, P. R., 1968 Biochemistry of differentiation. An. Rev. Biochemistry **37:** 631–660.

GROSS, P. R., K. KRAEMER and L. I. MALKIN, 1965 Base composition of RNA synthesized during cleavage of the sea urchin embryo. Biochem. Biophys. Res. Commun. **18:** 569–575.

HARRIS, S. E. and H. S. FORREST, 1967 RNA and DNA synthesis in developing eggs of the milkweed bug, *Oncopeltus fasciatus* (Dallas). Science **156:** 1613–1615.

KALF, G. F., 1964 Deoxyribonucleic acid in mitochondria and its role in protein synthesis. Biochemistry **3:** 1702–1706.

KRAUSE, G. and K. SANDER, 1962 Ooplasmic reaction system in insect embryogenesis. Adv. in Morphogenesis **2:** 259–303.

LIN, T. P., 1966 Microinjection of mouse eggs. Science **151:** 333–337.

LOCKSHIN, R. A., 1966 Insect embryogenesis: macromolecular synthesis during early development. Science **154:** 775–776.

MALKIN, L. I., P. R. GROSS and P. ROMANOFF, 1964 Polyribosomal protein synthesis in fertilized sea urchin eggs: the effect of actinomycin treatment. Develop. Biol. **10:** 378–394.

NUR, U., 1967 Reversal of heterochromatization and the activity of the paternal chromosome set in the male mealybug. Genetics **56:** 375–389.

PERRY, R. P., 1967 The nucleolus and the synthesis of ribosomes. Prog. Nucleic Acid Res. Mol. Biol. **6:** 220–257.

RITOSSA, F. M. and S. SPIEGELMAN, 1965 Localization of DNA complementary to ribosomal RNA in the nucleolar organizer region of *Drosophila melanogaster.* Proc. Natl. Acad. Sci. U.S. **53:** 737–745.

RITOSSA, F. M., K. C. ATWOOD and S. SPIEGELMAN, 1966 A molecular explanation of the bobbed mutants of Drosophila as partial deficiencies of "ribosomal" DNA. Genetics **54:** 819–834.

ROODYN, D. B. and D. WILKIE, 1968 *The Biogenesis of Mitochondria.* Methuen & Co., Ltd., London. Pp. 19–20.

SABOUR, M., 1969 Doctoral dissertation, University of California, Berkeley.

SCHRADER, F., 1923a The sex ratio and oogenesis of *Pseudococcus citri.* Z. ind. Abst. Vererb. **30:** 163–182. ——, 1923b The origin of the mycetocytes in *Pseudococcus citri.* Biol. Bull. **45:** 279–302.

SHINJI, G. O., 1919 Embryology of coccids, with especial reference to the formation of the ovary, origin and differentiation of the germ cells, germ layer, rudiments of the midgut, and the intracellular symbiotic organisms. J. Morphol. **33:** 73–168.