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EMBRYONIC DEVELOPMENT

Characteristic Features of Embryogenesis and Oogenesis in the Beetles *Adalia bipunctata* L. and *Harmonia axyridis* Pall. in All-Female Families

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Abstract—We describe abnormal embryonic development leading to death of 50% of embryos in all-female lines of *Adalia* and *Harmonia* infected with an androcide spiroplasma. The arrest of embryonic development takes place at different stages throughout embryogenesis. Oogenesis of the infected beetles proceeds without any significant morphological changes.

Key words: Coccinellidae, Adalia bipunctata, Harmonia axyridis, all-female state, embryogenesis, oogenesis.

In various groups of arthropods, microorganisms inherited cytoplasmically may lead to parthenogenesis, feminization, cytoplasmic incompatibility in crosses, and death of male embryos (O'Neil *et al.*, 1997). Such effects point to coevolution of the symbiont and the host, resulting in an interaction of the two organisms when the microorganism begins to control host reproduction.

Hurst (1993) has described two types of phenomena associated with male mortality: early mortality of embryos of the I instar larvae (found in many groups of insects and ticks) and late mortality of the IV instar larvae (found only in blood-sucking mosquitoes infected with microsporidia) (Chapman *et al.*, 1966; Hall, 1985).

The phenomenon of cytoplasmically inherited deviation from the normal sex ratio has been described for five species of ladybirds; this is associated with the death of male embryos at the stage of the egg (early mortality according to Hurst's classification) (Lus, 1947; Shull, 1948; Matsuka *et al.*, 1975; Hurst *et al.*, 1996). Recently, it has been confirmed that the all-female state in Coccinellidae is caused by the bacteria belonging to *Rickettsia* and *Spiroplasma* (Werren *et al.*, 1994; Balayeva *et al.*, 1995; Zakharov *et al.*, 1998). The morphopathology of male mortality in Coccinellidae has not been studied, and the state of the ovarioles of the infected females has not been described.

The purpose of the present study is to describe morphological manifestations of embryogenesis in all-female lines of *Adalia bipunctata* L. and *Harmonia axyridis* Pall., as well as the state of follicles in the infected females of these strains.

MATERIALS AND METHODS

We have used *Adalia bipunctata* L. (Coleoptera: Coccinellidae) beetles from St. Petersburg and Moscow populations and *Harmonia axyridis* Pall. (Coleoptera: Coccinellidae) from Novosibirsk; their females produced all-female offspring (no males were present). According to the results of nucleotide sequence analysis of a 16S rRNA gene fragment, all of the *A. bipunctata* beetles and one family of *H. axyridis* were infected

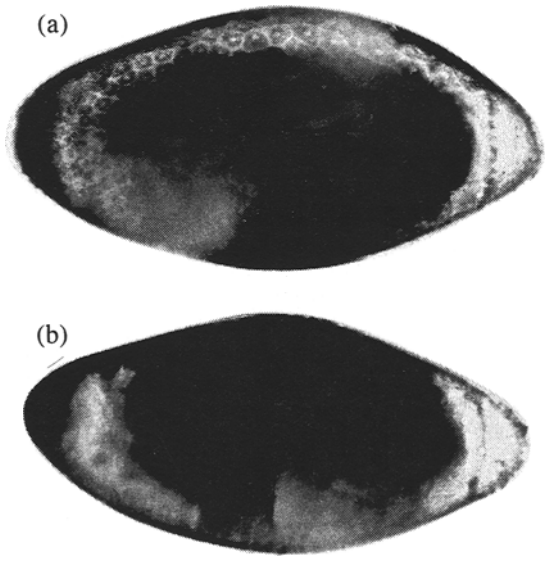

Fig. 1. The formation of the blastoderms during the first day of *Adalia bipunctata* development: (a) normal development; (b) developmental arrest. Magnification: ×40.

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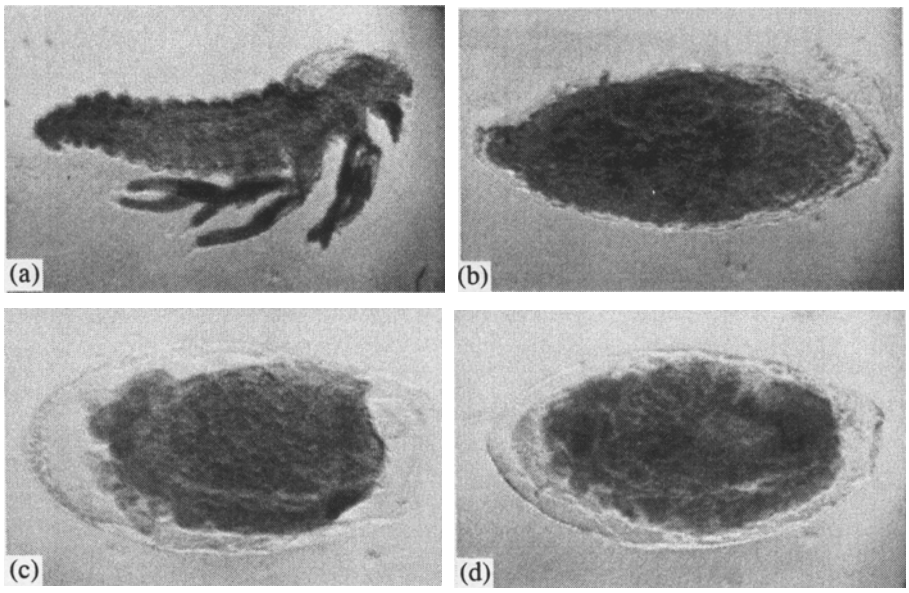

Fig. 2. Normal larva (a) and abnormally developing embryos at the stage of 72 h (b–d) in *Adalia bipunctata*. Magnification: (a) ×6.3; (b–d) ×16.

with *Spiroplasma* (Mollicutes) (Zakharov *et al.*, 1998; 1999).

The beetles were cultivated in the laboratory according to the techniques described earlier (Majerus *et al.*, 1989; Zakharov, 1995). Grain aphid *Scizaphis graminum* grown on wheat germs has been used as beetle food.

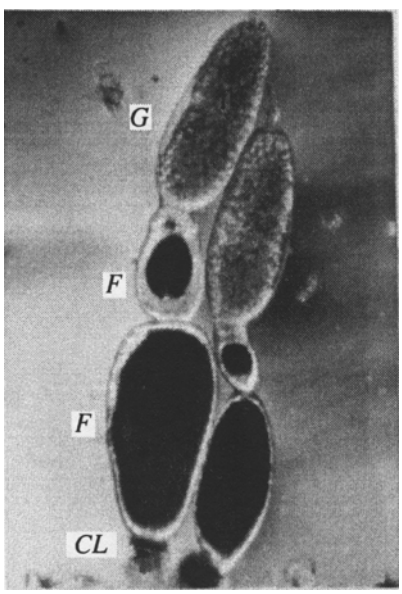

Fig. 3. General view of *Adalia bipunctata* ovarioles, staining with neutral red, magnification ×6.3. *G*, germarium, *F*, follicle; *CL*, *corpora lutea*.

In order to identify the all-female state of the offspring of the families analyzed, we observed the state of the eggs at the time of larvae emergence. If in several egg batches of one pair of beetles, the larvae were emerging from the egg membrane in about half of the eggs, the female of this pair operationally was considered as the carrier of a factor responsible for the all-female state. The sex ratio in the offspring was determined after the emergence of adult beetles from pupae on the basis of internal gonads.

Egg batches obtained from females which yielded all-female offspring were used in embryological studies, as well as egg batches from females with the normal sex ratio (control). The copulating beetles were placed in a separate Petri dish. Immediately after oviposition, the parents were transferred into another dish. Egg batches were kept at room temperature. We examined supravital preparations of embryos in paraffin oil ("hanging droplet") (dechorionated with a 10% aqueous solution of sodium hypochlorite), as well as fixed preparations stained with Azure-Eosine by the technique of Lillie and Zalocar (Lillie, 1954; Zalocar, 1971). The preparations of embryos embedded into Canadian balsam were examined under light microscopes manufactured by Karl Zeiss JENA (Germany) or Olympus (Japan).

Females with the confirmed trait of all-female offspring and females with a normal sex ratio in the offspring (control) were used for electron microscopy of the ovaries. The ovaries were fixed in a 2% glutaraldehyde made in phosphate buffer, as well as in a 1% osmium tetroxide. The material was dehydrated in alcohols, passed through acetone mixtures, and embedded into Epon-812. Sections were made with an LKB

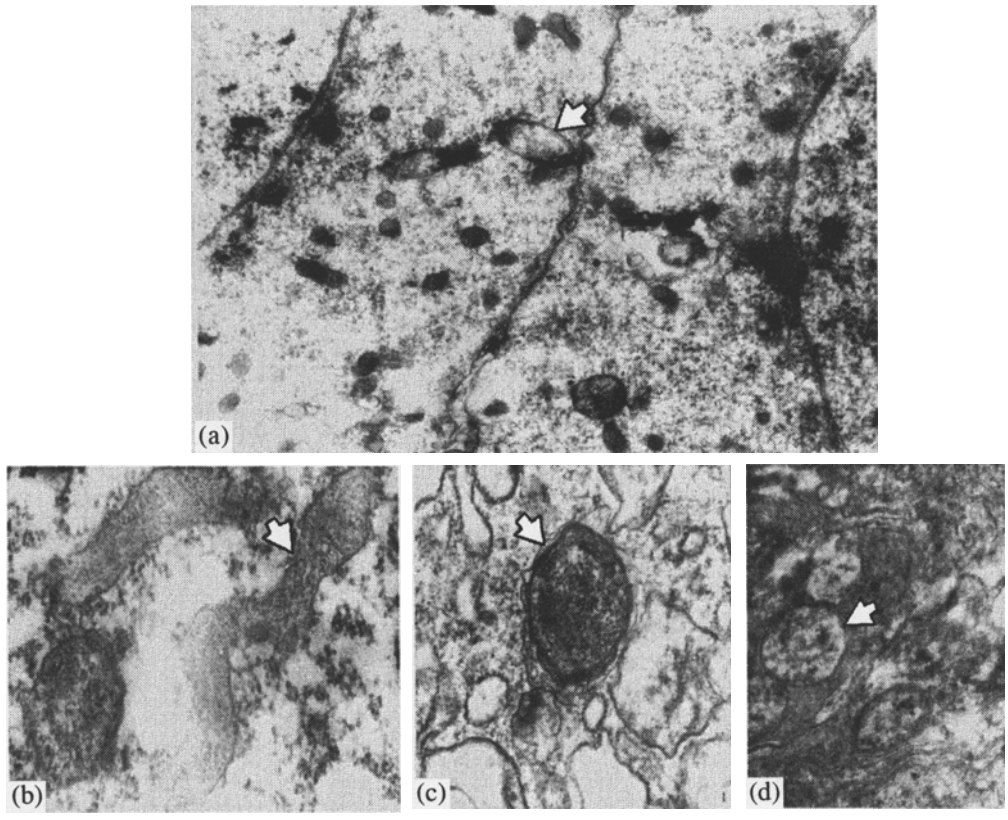

Fig. 4. Bacterial endosymbionts (arrows) in ovarioles of *Adalia bipunctata* (a, b) and *Harmonia axyridis* (c, d): (a) germarium, magn. ×21000; (b) oocyte, magnification ×100000; (c, d) follicular epithelium, magnification ×66000 (c) and ×52000 (d).

ultramicrotome, transferred onto grids coated with formvar film, and post-stained with 2% uranyl acetate and 2% lead nitrate. The preparations were viewed in a JEM-100CX electron microscope (Japan) at an accelerating voltage of 80 kV.

RESULTS

Embryogenesis. The embryos of all strains used in the experiments with the trait of all-female offspring gave a similar result (with one exception in *Harmonia*). By observing the stage of eggs with arrested development after the other eggs produced larvae, we found that these eggs generally retain their yellow color and shape for 2–3 days after the emergence of the larvae from other eggs of a given batch. At the early stage of embryogenesis, we did not observe any deviations in the development of the embryos. In about 1/8 of embryos, the arrest of development was seen after the first day of embryogenesis (Fig. 1). Normally at this period, blastodermal cells segregate, the embryonic streak is formed, and rudiments of extremities are produced. Abnormal embryogenesis at this period appeared as developmental arrest at the stage of early blastoderm or during the first phase of gastrulation. In another 1/8 of the embryos, the arrest of development was seen before the stage of 45–50 h. At the stage when larvae were emerging from the pupae (about 72 h in *Adalia bipunctata* and 96 h in *Harmonia axyridis*), morphological abnormalities in such embryos corresponded to different periods of development, and the necrotic processes had different degrees (Fig. 2). Embryos whose development had stopped at different times were similar to normally developing embryos but at earlier developmental stages.

In one line of *Harmonia axyridis*, we found a pattern of embryonic development that differed from that described above for the all-female strains of *Adalia bipunctata* and *Harmonia axyridis*. Generally, individual embryos (in some egg batches, up to 30% of embryos) did not develop at all; half of the embryos, however, developed normally (later they yielded females), and the remaining part of the embryos continued development to the late embryonic stage but did not produce larvae.

Oogenesis and the bacterial infection of follicles. The thelotrophic ovarioles of reproducing beetles of both studied species consist of a germarium and two vitellogenic follicles (Fig. 3). The yellow bodies can be seen at the basis of the ovarioles in ovaries of depositing females. The morphology of the developing follicles studied with the aid of the light microscope does not differ in the infected and control females.

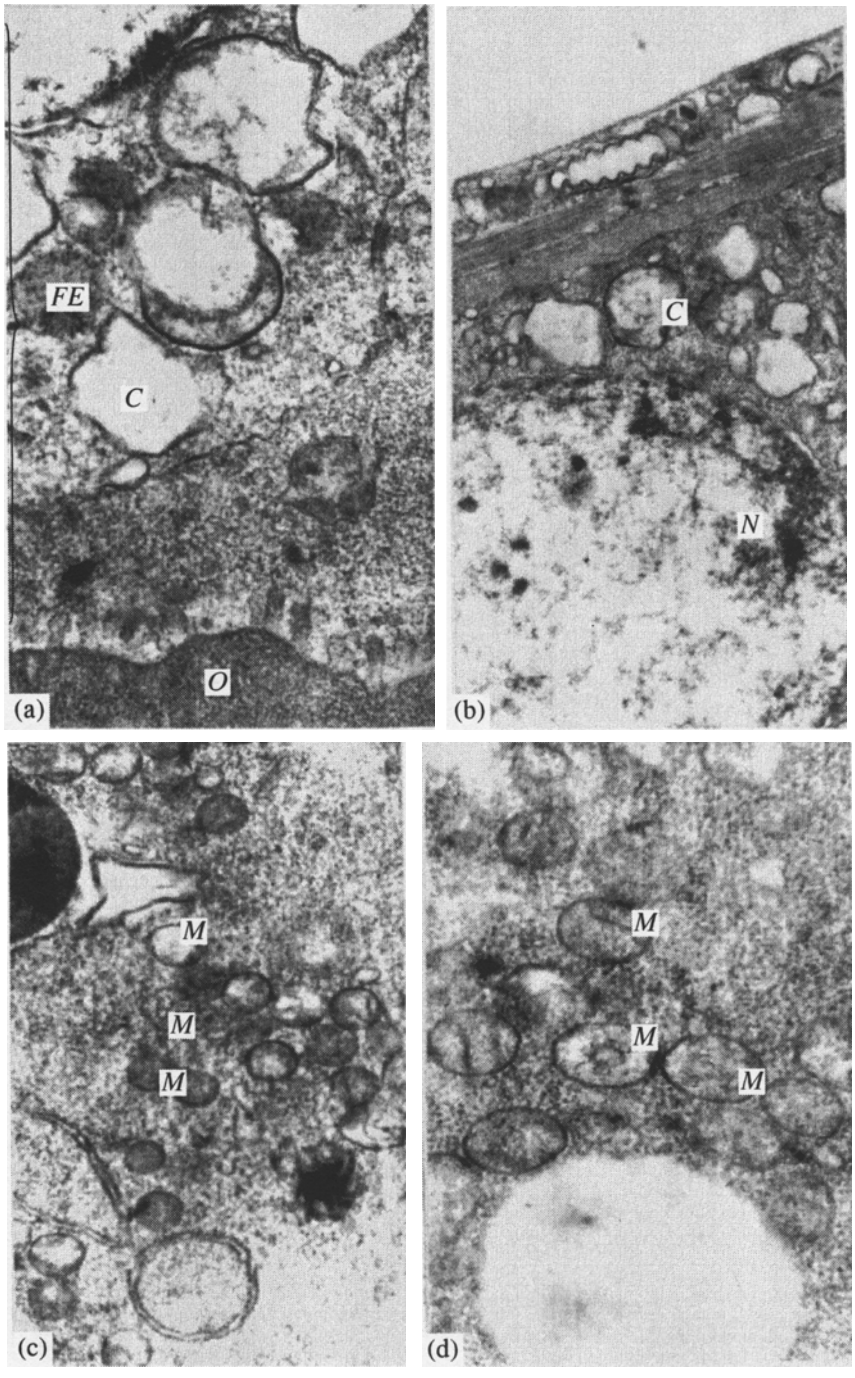

Fig. 5. Follicular epithelium (a, b) and oocyte (c, d) of infected (c) and control females (a–c) in *Harmonia axyridis*. Magnification: ×40000 (a); ×20000 (b); ×32000 (c); ×66 000 (d). *M*, mitochondria; *O*, oocyte; *FE*, follicular epithelium; *C*, cytoplasmic cysterns; *N*, nucleus.

The electron-microscopic examination allowed us to identify microorganisms in the cytoplasm of germarium cells (Fig. 4a). Two more types of microorganisms have been found in the follicular epithelium: They were present in the cytoplasm (Fig. 4b) and intercellular spaces (Fig. 4c). The oocytes have been found to contain helix-like spiroplasma-like miroorganisms (Fig. 4d). All microorganisms found have different morphologies. The normal cellular cytoplasmic structure of the follicular epithelium in oocytes are shown in Fig. 5. We assume that only microorganisms found in intercellular spaces of the follicle epithelium and in the oocyte (Figs. 4b, 4c) can correspond to the coccal and helix-like forms of the spiroplasma. We did not observe

any massive bacterial infection of the follicles. No endosymbionts have been found in the ovarioles of noninfected females (Fig. 5).

DISCUSSION

The embryology of insects infected with microorganisms which affect the sex ratio has been studied in detail in various strains of *Drosophila*. The developmental arrest and death of males generally takes place at different stages of embryogenesis (Counce and Poulson, 1966; Tsuchiyama-Omura *et al.*, 1988). When the embryo was infected, the greatest damage was seen in the nerve tissue.

In all-female families of *Adalia bipunctata*, the mortality of the male embryos has been recorded at the stage of the egg (Lus, 1947). The results of our study have demonstrated that the factor of the male-free state does not preclude the beginning of cleavage and that the development of the embryos stops at various stages of organogenesis. These results lead to the conclusion that the presence of a Y chromosome is not a primary signal for bacteria to immediately "destroy" the male embryo.

The embryogenesis of the infected beetles has certain sensitive periods, or "development gates," which are closed for a certain number of male embryos, while the female embryos and a part of the male embryos pass successfully through these gates. There may be at least four such periods of embryogenesis critical for the male embryos.

In *Harmonia axyridis*, we found a family whose male offspring were dying at the stage of the late embryo. Such embryos removed from egg membranes had a characteristic muscular dystrophy.

When adult blood-sucking mosquitoes were experimentally infected with spiroplasma, extensive degradation of the wing musculature was identified, and bacterial cells were found in numerous tissues and in hemolymph (Philips and Humphery-Smith, 1995). These authors have found several types of bacteria. Our observations allow a conclusion (with some caution) that spiroplasms can be polymorphic in the studied beetle species. In *Drosophila*, we have found only one way of oocyte infection with the spiroplasma during ontogenesis on the basis of intercellular spaces of the follicular epithelium (Niki, 1988). As shown by our observations, this may be the only way of passage of the spiroplasms responsible for the transovarial transmission of bacteria into the insect oocyte.

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