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Bacteria in ovarioles of females from maleless families of ladybird beetles *Adalia bipunctata* L. (Coleoptera: Coccinellidae) naturally infected with *Rickettsia*, *Wolbachia*, and *Spiroplasma*

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Abstract

Ovarioles were found to be infected with *Spiroplasma*, *Wolbachia*, and *Rickettsia* in *Adalia bipunctata* females with maleless progeny in different natural populations. Ooplasm was infected with few *Wolbachia* bacteria. In ooplasm infected by *Rickettsia*, bacteria were present in small foci. Spiroplasmas were found encapsulated into ooplasm from the wider intercellular spaces between epithelial and oocyte cells. The cytoplasm of follicular epithelia infected with *Rickettsia* was heavily destroyed, but the nucleus was intact and free from bacteria. The essential feature of follicular epithelium cells from *Spiroplasma* and *Wolbachia* infected *A. bipunctata* females was inclusions of three types: crystalline, filaments, and concentric myelin-like lamellae. Observations of smears prepared from ovaries of *A. bipunctata* from natural populations revealed a low concentration of bacteria within a microscopy field (less 10 bacteria) in more than 90% of specimens, and only a few ovaries were heavily infected. Two different ways of bacterial invasion of the oocyte are suggested: Spiroplasma-like, through the intercellular spaces in the epithelium and Rickettsia-like, through the cytoplasm of follicular epithelium cells. Bacteria were not found in germarium zones and we suggest that each follicle is infected from haemolymph.

Keywords: *Adalia bipunctata*; Ovarioles; Cell inclusions; Sex-ratio distortion; Maleless progeny; Bacteriae; *Rickettsia*; *Spiroplasma*; *Wolbachia*; Transmission electron microscopy

1. Introduction

In arthropods, female-biased sex ratios due to mortality of male embryos and cytoplasmic incompatibility causing high embryonic mortality of both sexes are two examples of how eukaryotic embryological mechanisms are controlled by prokaryotes. At present three bacterial genera are confirmed to be involved in such mechanisms: *Rickettsia*, *Spiroplasma*, and *Wolbachia* (Balayeva et al., 1995; Ebbert, 1995; Werren et al., 1994, 1995). Transovarial transmission of cytoplasmically inherited genetic factors (bacteria) causing male-killing trait in arthropods is well documented (see review of Hurst, 1993).

Microorganisms suspected as sex-ratio distorters are found living in haemolymph as *Spiroplasma* in *Drosophila* or invading haemocytes as *Rickettsia* in *Adalia* (Hurst et al., 1996; Niki, 1988). In *Drosophila melanogaster*, Spiroplasmas invading ovarioles from the haemolymph first break the basement membrane (tunica propria) of a follicle. They then move along the intercellular space between the follicular epithelium cells at the previtellogenic stage and on reaching the oocyte are incorporated into ooplasm by pinocytoses (Niki, 1988). Then Spiroplasmas in maturing oocytes are usually found in yolk granules and vesicles until oviposition (Niki, 1988). Detailed information on the mechanism employed by *Rickettsia* to infect insect ovarioles is absent in systems with maleless trait.

In *Adalia bipunctata*, the object of the present study, three bacteria are reported to cause male-killing trait in

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different populations: *Spiroplasma*, *Wolbachia*, and *Rickettsia* (Hurst et al., 1999a,b; Werren et al., 1994; Zakharov et al., 1998; Zakharov et al., 2000). In this paper, the bacteria infected ovarioles are described on transmission electron microscopy level.

2. Materials and methods

2.1. Strains of ladybird beetles and their maintenance

We used different stocks of *A. bipunctata* strains both infected and uninfected by male-killing bacteria. The first *Rickettsia*-infected line was separated by one of us (I.A.Z.) from the natural Springfield population (UK) which was kindly provided by Dr. M. Majerus (strain SP4). *Spiroplasma* infected lines were from Saint Petersburg, Russia (PF4) and Tuva (TyV_2); molecular identity of the male-killers has been confirmed (Zakharov et al., 1998). Lines I22 from Moscow and T17 from Tomsk carried a *Wolbachia* infection (Zakharov et al., 2000). For the aim of comparison, *Harmonia axyridis* Pall. from Novosibirsk (Russia) (strain Novo 108) was also studied. As controls insect families with normal sex ratio were used.

The strains were maintained in the laboratory as it is described earlier (Zakharov, 1995); using aphids *Sciza-phis graminum* as food to produce eggs and fresh banana chips for keeping beetles alive during some periods. Maleless lines were established by crossing infected females with normal males and identified in the F_1 generation by low egg hatch-rate (about 50%) and progeny sex-ratio analysis (Hurst et al., 1994).

2.2. Preparation for electron microscopy

Ovaries from females were carefully extracted with needles in a drop of PBS. Usually, one ovary was checked for bacterial identification by PCR (following the standard procedure, Sambrook et al., 1989), the other was quickly passed into 2% glutaraldehyde for 3 h. Ovaries were washed in PBS and post-fixed with 1% osmium tetroxide for 1 h. Then staining en bloc with 2.5% uranyl acetate was performed. The material was then dehydrated in a series of ethanol solutions up to the absolute ethanol. Following immersion in acetone and mixture of acetone and resin, the materials were embedded in Epon 812. Polymerisation was carried out in an oven at 37 °C for 2 days and then 60 °C for 2 days. Sections of 90 mm were cut on LKB microtome. The sections were placed on copper grids covered with formvar film and stained in for 15 min in each of 2.5% uranyl acetate and 2% lead citrate. The material was observed at 80 kV on a JEM-100CX electron microscope.

2.3. Light microscopy

Light microscopy was used for observing the gross morphology of ovarioles (objectives 6.3×–40×), and in addition ovary squashes stained with methylene blue were inspected for bacteria-like organisms (objective 90×, oil immersion); ovarioles in ovaries were counted (objective 2×). Preparations were observed under a Fluoval (Karl Zeiss JENA) microscope. Ovarioles were photographed with 400 ISO Kodak film.

3. Results

3.1. Gross morphology of ovaries and ovarioles

Adult female *A. bipunctata* ladybird beetles posses two ovaries with telotrophic ovarioles (Fig. 1). Each ovariole consisted of a germarium and one follicle in reproductively active females, and only of a germarium in young females or those maintained on banana in refrigerator for a long period.

In the germarium of another coccinellid, *Coccinella septempunctata* L., the differentiation of oocytes and trophocytes occurs in larvae of "last stage" (Kozhanova and Pasychnik, 1979). In reproductively active adult females *A. bipunctata*, we found no developmental delays or morphological abnormalities in follicles.

The number of ovarioles was quite stable within studied populations in different years. Mean numbers varied little—27–30 ovarioles in both ovaries (Table 1). Females usually oviposit a batch of 12–30 eggs. After oviposition, the corpus luteum (yellow body, Fig. 1) is visible at the base of ovarioles and may serve as a criterion of parous status.

The smears of ovarioles revealed differences in the density of bacteria-like organisms within two natural populations. More than 90% of preparations had 1–10 bacteria-like organisms spread evenly throughout any field of view (magnification 90×, oil immersion), less than 10% had 10 and more stained bacteria. A few preparations had even higher burdens. Oval and round shape of two sizes bacteria were found in St. Petersburg and Moscow populations. The main criterion of developing infection was existence of dividing bacteria. The total infection levels in two populations are presented in Table 2.

We have also investigated a few preparations from an *A. bipunctata* population in Kirgisia with presumably Rickettsial infection. We found only rod-shaped bacteria-like organisms in ovariole smears (Ki11). But we did not find bacteria in electron microscopy preparations from Ki3 strain (Fig. 2). In smears of *H. axyridis* from Novo 108 families (Novosibirsk), we found a different pattern of bacterial distribution within preparations: few very compact foci of oval microorganisms. In the

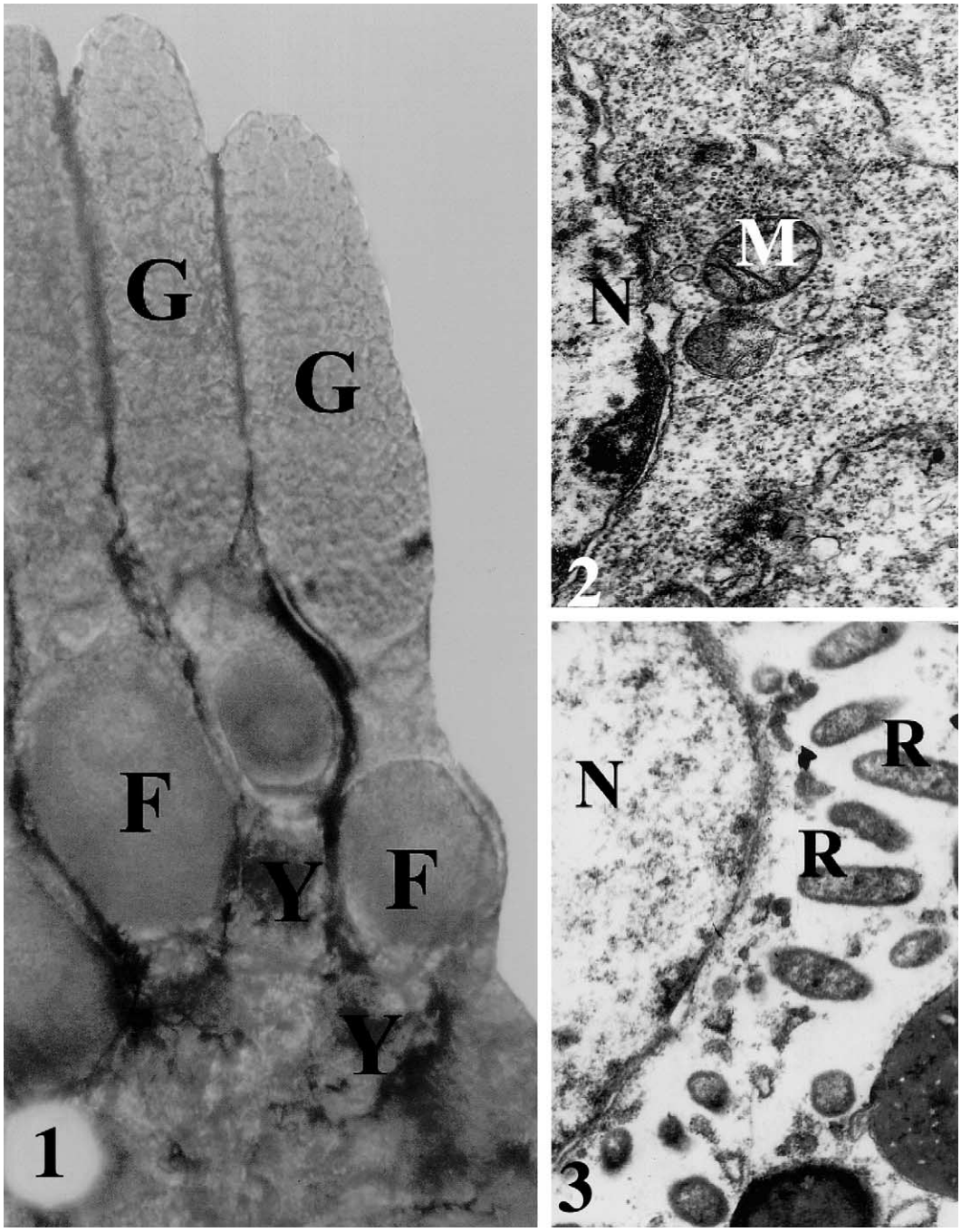


Fig. 1. Ovarioles of parous *A. bipunctata*. Supravital preparation in neutral paraffin oil. G, germarium; F, follicle; Y, corpus luteum. Total magnification is 80×.
Fig. 2. Follicular epithelium cell of *A. bipunctata*, maleless strain Ki3. N, nucleus; M, mitochondria. Total magnification is 27,000×.
Fig. 3. Follicular epithelium cell, infected with *Rickettsia*. *A. bipunctata*, maleless strain SP4. R, *Rickettsia*; N, nucleus. Total magnification is 16,600×.

Table 1
Ovariole number of *A. bipunctata* in natural populations in Russia; standard errors of means are given in parentheses

Population	Year	Mean number of ovarioles	Number of specimens	Size of femur (mm)
Moscow	1996	30 (0.70)	93	1.42 (0.01)
Moscow	1997	28 (0.47)	80	1.46 (0.01)
St. Petersburg	1996	28 (0.40)	94	1.31 (0.01)
St. Petersburg	1997	27 (0.34)	103	1.40 (0.01)

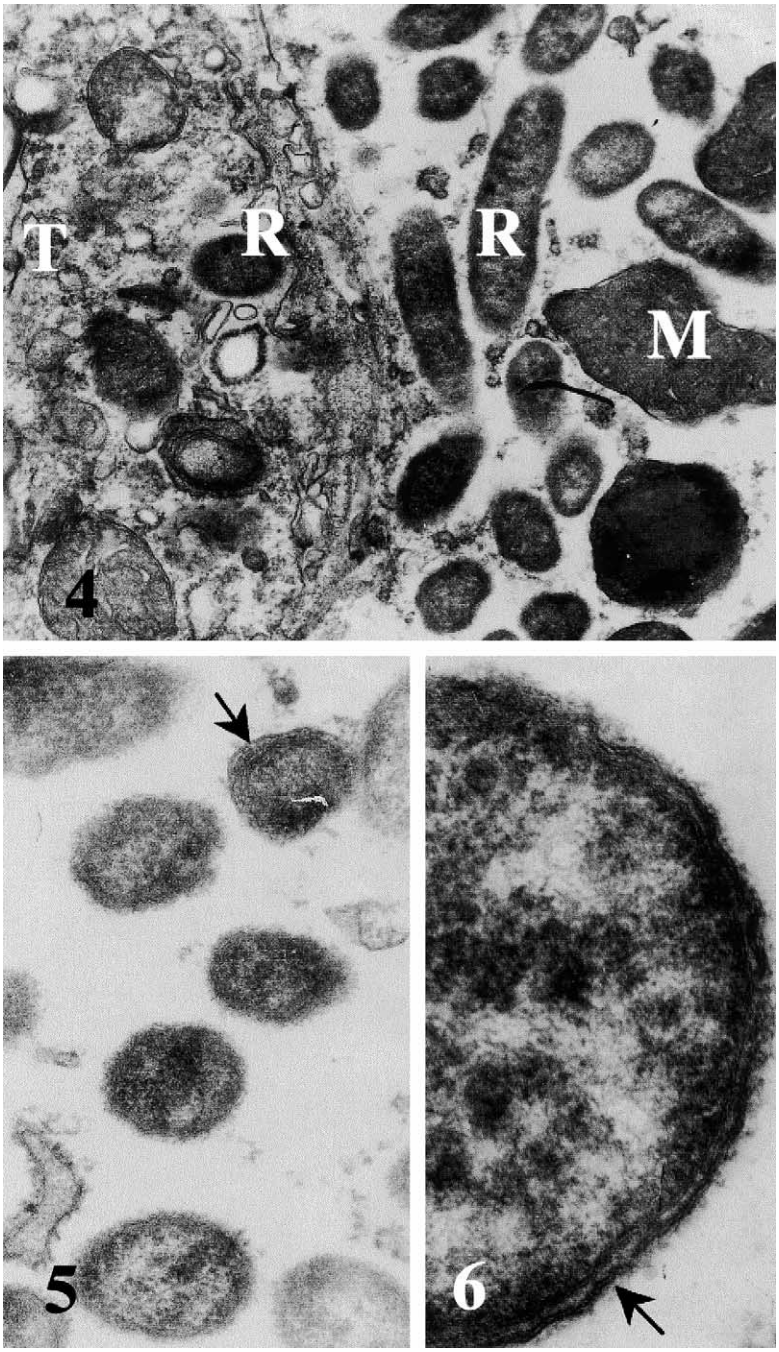


Fig. 4. Tracheolar cell (T) adjacent to follicular epithelium cell (F). *A. bipunctata*, maleless strain SP4, infected with *Rickettsia*. R, *Rickettsia*; M, mitochondria. Total magnification is 32,500×.

Fig. 5. Transverse cross-sections of *Rickettsia* from *A. bipunctata*, maleless strain SP4. Clear visible layer walls (arrow). Total magnification is 66,000×.

Fig. 6. Cross-section of *Rickettsia* from *A. bipunctata*, maleless strain SP4. Total magnification is 125,000×.

Table 2
Bacteria-like infection in smears of ovarioles of *A. bipunctata* from natural populations in Russia (standard errors of means are given in parentheses)

Population	Number of females	% of infected preparations
Moscow, 1997	80	12.5 (3.7)
St. Petersburg	99	47.5 (5.0)

Novosibirsk *H. axyridis* population, the *Spiroplasma* infection was confirmed by PCR (Zakharov et al., 1999). This method of staining ovary smears may provide a rapid method to asses infection prevalence in populations. The results of the smear method may explain why previous studies were failed to find bacteria in ultrastructural studies of ovarioles infected with low number

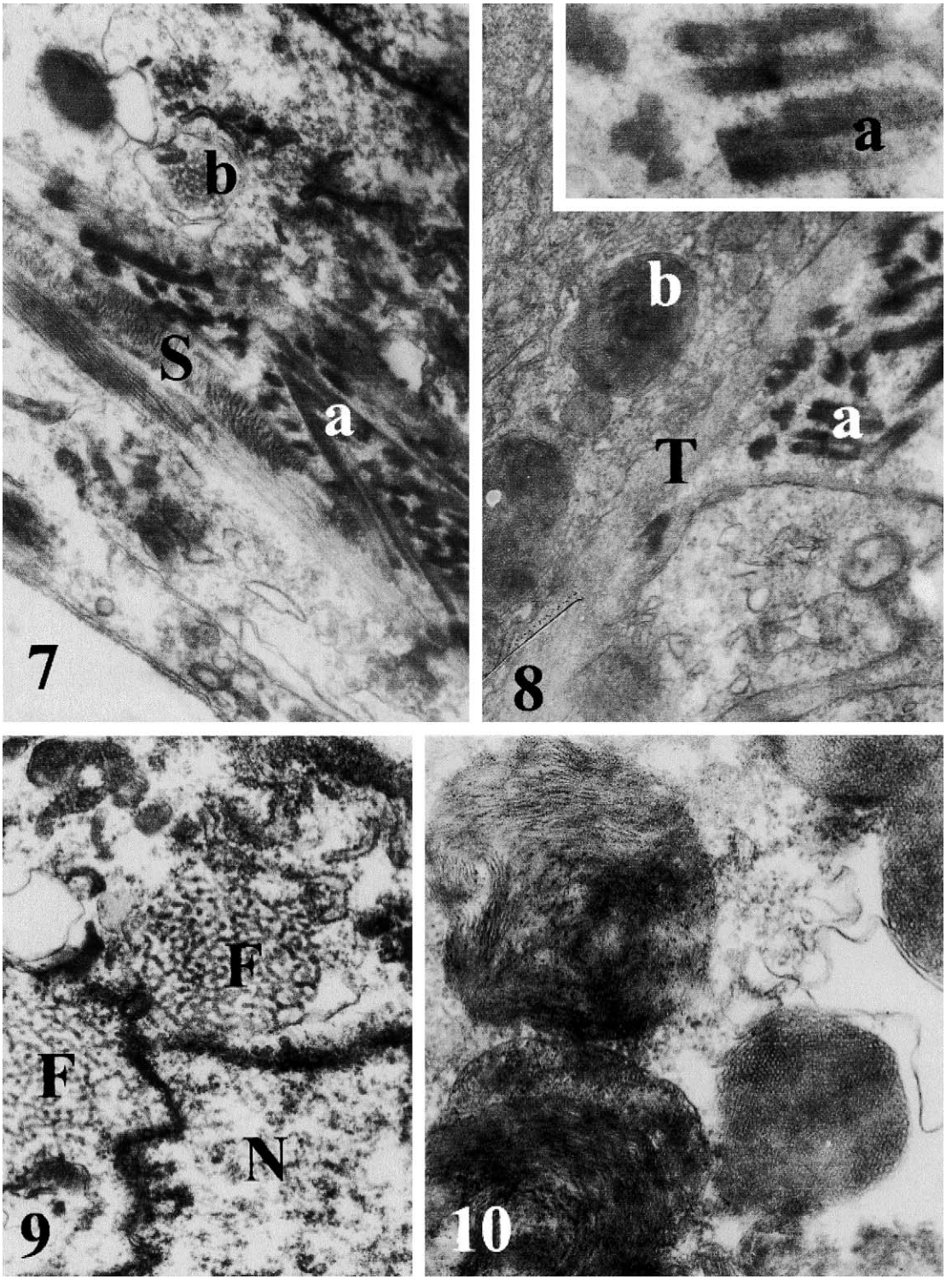
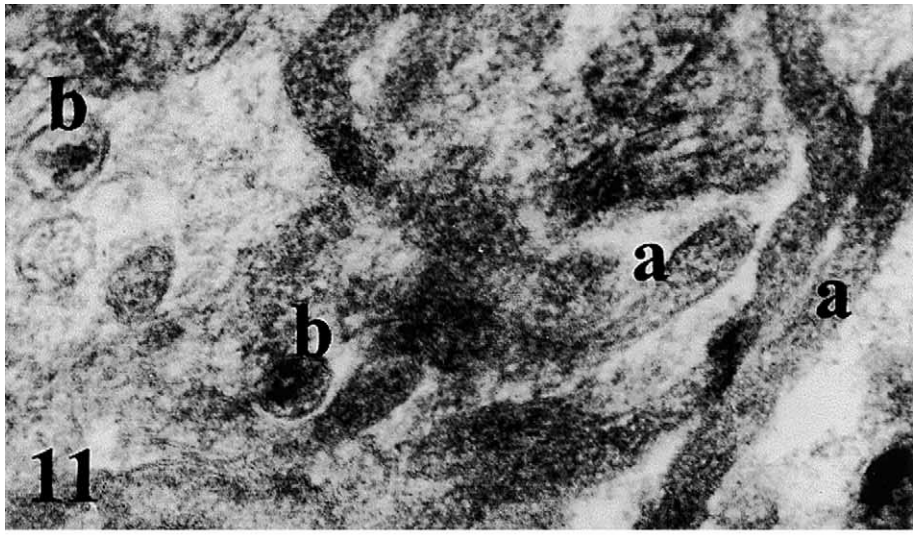


Fig. 7. Crystalline (a) and filamentous (b) inclusions in follicular epithelium from *A. bipunctata* (I22), maleless strain with *Wolbachia* infection. S, ovariolar sheath. Total magnification is 19,500×.

Fig. 8. Crystalline (a) and concentric myelin-like lamellae (b) in follicular epithelium from *A. bipunctata*, maleless strain PF4 with *Spiroplasma* infection. T, tunica propria (basal membrane). Total magnification is 25,600×, high magnification inclusion is 85,000×.

Fig. 9. Locally concentrated filaments (F) in follicular epithelium in vicinity of nucleus (N). *A. bipunctata*, maleless strain I22 with *Wolbachia* infection. Total magnification is 30,000×.

Fig. 10. Concentric myelin-like lamellae in follicular epithelium from *A. bipunctata*, maleless strain PF4 with *Spiroplasma* infection. Total magnification is 39,000×.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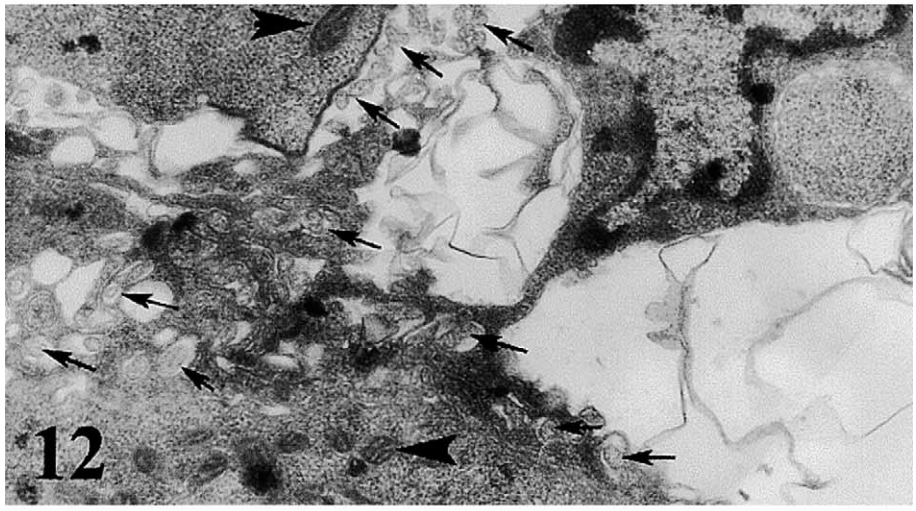
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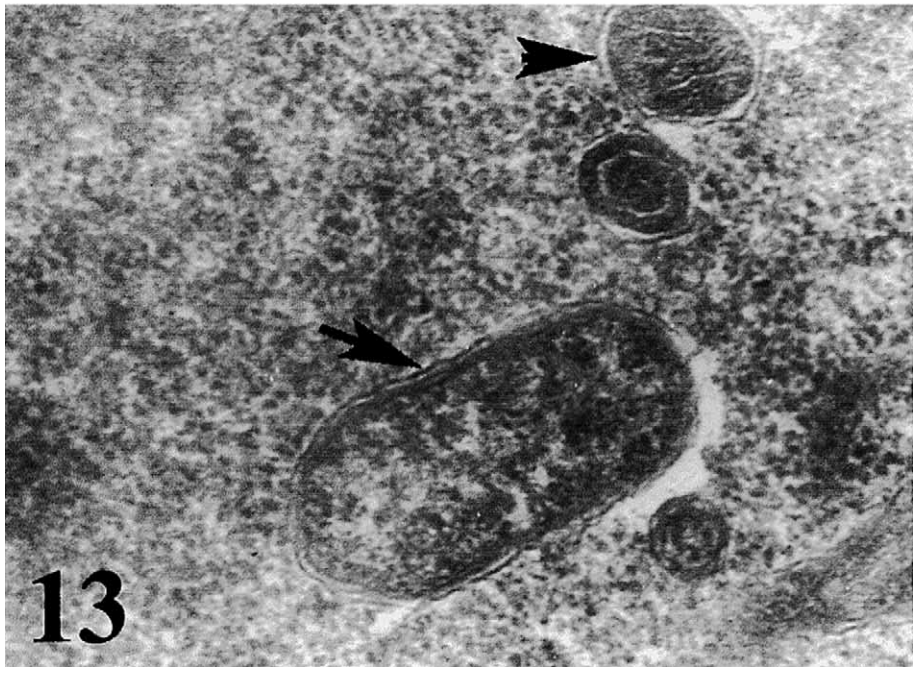
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of *Spiroplasma* or with infection in only the haemolymph (see review Hurst, 1993).

3.2. Follicular epithelium features

Adalia bipunctata from the English line SP4 had severe Rickettsial infection in the cytoplasm of follicular epithelium cells and foci of different density in the ooplasm (Fig. 3). The bacterial morphology is similar to those of bacteria described from haemocytes from the similar laboratory line in Cambridge (Hurst et al., 1996). We observed that the bacteria almost entirely destroy the cytoplasm of the epithelial cell, but the nucleus is intact and free from bacteria. Occasionally, we observed *Rickettsia* in tracheolar cells (Fig. 4). High magnification (60,000×, 100,000×) revealed the poly-layer structure of bacterial wall (Figs. 5 and 6).

Ovaries embedded en bloc (30 in total) from families or females originating from Moscow infected with *Spiroplasma* or *Wolb* were studied. In all cases the follicular epithelium cytoplasm lacked bacteria. However, the infection caused three different types of inclusions. First, crystalline inclusions (Figs. 7a and 8a) were usually found in the space under the ovariolar sheath, near the follicular epithelium. Some were incorporated into cytoplasm, and some were visible piercing the basal membrane of a follicle. These inclusions were always found in clusters (not scattered in the cytoplasm). High magnification (85,000×) did not reveal any internal structures inside them (Fig. 8, high magnification inclusion). Second, in the *Wolbachia* (Moscow) infected line, we observed prominent filaments clustered in high concentrations. They formed an anastomising group in vicinity of the basal membrane (Fig. 7b) or nuclear membrane (Fig. 9). Sometimes these filaments were visible inside vacuoles. The physiological significance of this local increase in the concentration of these elements is not understood. Third, concentric systems of thin lamellae were visible in inclusions of the cytoplasm of follicular epithelial cells in line PF4 (Figs. 8b and 10). In Ki3, *Rickettsia* infected similar structures were found inside vesicles. They may be interpreted as myelin forms of hydrated phospholipids. Such inclusions were routinely observed in epithelial cells (Fawcett, 1981). We suggest these structures may be a result of multiple invasions of pathological agents into cells.

The *Spiroplasma*-like structures were found in sections from line PF4 (Fig. 11). They were not clearly helical but similar to those reported by Niki (1988) (Fig. 5 insertion). They were oval and elongated structures without a cell wall, characteristic ultrastructure for this microorganism. These were found in intercellular spaces and inside vesicles close to cell borders in the follicular epithelium and in the ooplasm of *A. bipunctata* (Fig. 12) from the Moscow population.

Wolbachia sp. were found mostly as single or doubled cells around the ooplasm in *Adalia* oocytes in infected families from T17 and I22 (Fig. 13). All bacterial cells were incorporated into vacuoles with characteristic three delimiting membranes (the third is from a vacuole).

4. Discussions

At least two conclusions relevant to the mechanisms of bacterial invasion into a follicle are evident from the results of the study. First, *Rickettsia* initially occupies the follicular epithelium and then the oocyte of follicles in *A. bipunctata*. Second, *Spiroplasma* occupies the borders of the follicular epithelium cytoplasm and oocyte ooplasm in the species. This may give indirect support to the model of *Spiroplasma–D. melanogaster* interaction described by Niki (1988) who proposed that *Spiroplasma* invade a host oocyte through the intercellular spaces. We suggest two different trans-ovarial transmission mechanisms are employed by these bacteria. In addition, we want to stress that the bacteria are intended to infect each new follicle rather than be passed from germarium to the inside oogonial cells.

Different species of *Spiroplasma* have several morphological forms sometimes associated with viruses in various arthropods (Niki, 1988; Philips and Humphery-Smith, 1995; Tully et al., 1995). Until now the effect on hosts of each form is not clear. *Wolbachia* in maleless *Adalia* appears quite similar in its ultrastructure to *Wolbachia* from other arthropods (O'Neill et al., 1997).

Rickettsia and *Wolbachia* belong to the same family Rickettsiaceae. However, the behaviour of bacteria of two genus are different. We found that number of bacterial cells in the cytoplasm of infected insect cells differ depending on the bacterial species. We found a large quantity of *Rickettsia* and only single *Wolbachia* cells in the cytoplasm of infected *Adalia* cells (see Figs. 3 and 13).

We confirmed by electron microscopy the presence of three different bacteria in ladybirds of Russian popula-

Fig. 11. Longitudinal (a) and transverse (b) sections of *Spiroplasma*-like organisms from follicular epithelium of ovarioles of *A. bipunctata*, maleless strain PF4. Total magnification is 100,000×.

Fig. 12. *Spiroplasma* (arrows) in intercellular spaces and in vesicles in the edge of ooplasm of *A. bipunctata* follicles (strain TuV_2). Arrowheads point to mitochondria. Total magnification is 70,000×.

Fig. 13. *Wolbachia* sp. (arrows) from ooplasm of *A. bipunctata* follicles (strain T17). Arrowheads indicate mitochondria. Total magnification is 20,000×.

tions performing the same male-killing trait. Accumulating scientific information will allow us to establish the models of transovarial transmission of these bacteria affecting reproduction strategy of arthropods.

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