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Histopathological Investigations on *Rickettsiella*-like sp. and Nonoccluded Viruses Infecting the Pecan Weevil *Curculio caryae,* the Squash Beetle *Epilachna borealis,* and the Mexican Bean Beetle *Epilachna varivestis*

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Electron microscopic observations of abnormal specimens of pecan weevils, *Curculio caryae,* squash beetles, *Epilachna borealis,* and Mexican bean beetles, *Epilachna varivestis,* obtained from insect-rearing facilities and from field collections revealed that rickettsia-like organisms (RLOs) and viruses caused disease in these species. Several pleomorphic forms of the RLOs which were similar to those of *Rickettsiella* sp. were found in tissues of the fat body, hypodermis, tracheal matrix, muscle, and midgut of each insect species. In addition, RLOs also were found in heart and silk gland tissues of the pecan weevil. Stalked knobs or "pili" were found on the cell membranes of the RLOs in the infected tissues of the pecan weevil and the squash beetle. Crystals often associated with *Rickettsiella* infections were not observed. Virus-like particles of 18–42 nm were found in various tissues of the three species examined.

KEY WORDS: ***Rickettsiella*-like organisms; virus-like particles; ultrastructural investigations; pecan weevil; squash beetle; Mexican bean beetle.**

INTRODUCTION

Outbreaks of disease occur in the field and in insect-rearing facilities. In many cases viruses or fungi are often the primary pathogens involved. The purpose of this report is to describe rickettsia and viruses causing diseases in the pecan weevil *Curculio caryae,* the squash beetle *Epilachna borealis,* and the Mexican bean beetle *Epilachna varivestis.*

MATERIALS AND METHODS

From 1978 to 1984 we received many insects for disease diagnoses and routinely observed rickettsia-like organisms. The pecan weevils were received from the insect-rearing facilities at Mississippi State University. The squash beetles were collected at the USDA, ARS quarantine facilities at Newark, Delaware, and the Mexican bean beetles were collected in the field at several Maryland locations. Tissues were taken for light (LM)- and electron-microscopic (EM) examinations and fixed in Bouin–Duboscq fixative (LM) or 2.5% glutaraldehyde in 0.05 M cacodylate buffer (EM) according to previously described procedures (Adams and Bonami, 1991). Sections for LM were stained with Giemsa and Hamm (1966) stains. Ultrastructural examinations were made with a Philips 301 or 400 T[1] electron microscope. Measurements of the pathogens were made from the sectioned material.

RESULTS

Pecan weevils. Infected larvae were often swollen and lethargic with necrotic spots on the cuticle. A stereoscopic microscopic examination of diseased pecan weevil larvae revealed necrotic spots on the heart lying directly below the dorsal cuticle. In the dissection process, we found necrotic spots on Malpighian tubules, silk glands, and trachea, but in no case was there extensive damage to any tissue except the fat body which was lysed. EM examinations revealed *Rickettsiella*-like organisms (RLOs) in the midgut and the muscle and trachea surrounding it, the heart, Mal-

† Deceased.

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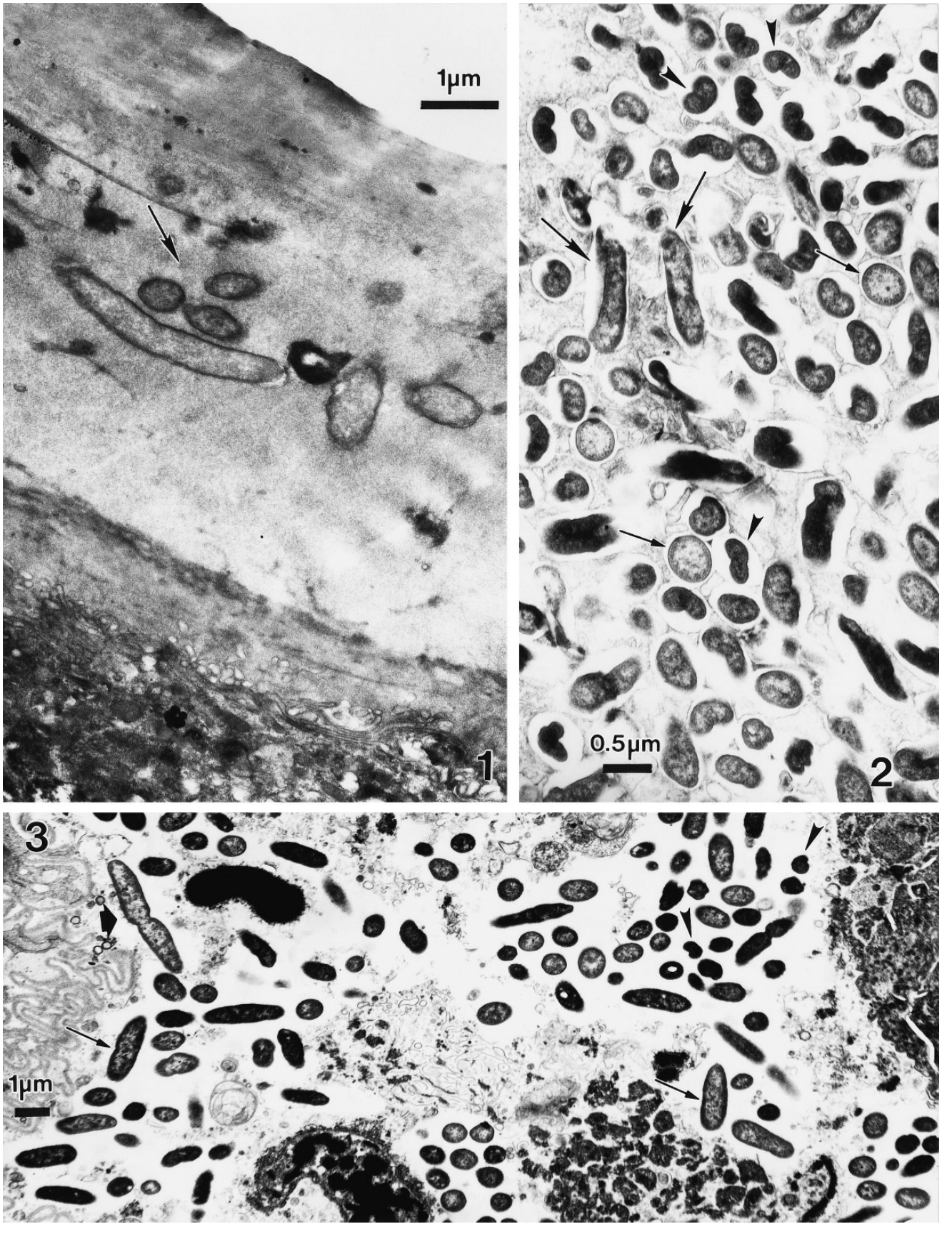
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pighian tubules, hypodermis, muscle, and tracheal matrix. Figure 1 shows some of the RLOs that were trapped in the cuticle during a molt. The fat body cytoplasm was completely degenerated. The "healthy" appearing pecan weevil larvae were in fact infected but had lighter infection sites than the tissues examined in the "diseased" larvae. The RLOs were pleomorphic with small condensed bodies (elementary bodies or "standard cells") measuring approximately 0.19–0.27 × 0.41–0.48 or 0.2–0.3 × 0.4–0.5 μm. Some "cells" appeared to be kidney-shaped while others were coccoid and dividing and others were small rods (Fig. 2). In vacuoles or lacunae of the host cells, the RLOs were enlarged to a less condensed form. These bacilliform bacteria or "secondary cells" measured approximately 0.3–0.5 × 1.85–3.2 μm and divided by binary fission. One section of the heart contained microorganisms which occurred singly rather than in vacuoles and these cells measured 0.4–0.6 × 0.8–2.6 μm, slightly larger than those observed in other infected tissues. Crystal formation similar to that which occurs in *Rickettsiella popilliae* multiplication or in *R. melolonthae* was not observed. In addition, we found a spherical nonoccluded virus of 42-nm diameter in the heart tissue and developmental forms of virus-like particles (VLPs) (38–40 nm in diameter) replicating in the cytoplasm of the tissues associated with the heart (not shown). Examinations of muscle sheath tissues near the hindgut also revealed small vesicles containing VLPs of the same diameter (38–40 nm). Within the muscle, VLPs of approximately 18 nm were replicating in areas where degeneration of the muscle fibers had begun (not shown).

Squash beetles. The squash beetles were being reared in the Newark, Delaware, lab for parasite studies. "Sick" fourth instar larvae dropped off the squash plants, laid on their backs, and mummified. In addition to the lethargy observed, necrotic spots occurred on some of the spines. Dissection of the tissues revealed only a darkened area on the midgut of one of the larvae. Electron-microscopic examination revealed pleomorphic RLOs in the gut, muscle, trachea, and hypodermis. The "secondary cells" were 0.5–0.7 μm in diameter × lengths up to 2.5 μm while smaller coccoid forms or "standard cells" measured approximately 0.4–0.6 × 0.7–1.2 μm (Figs. 3 and 4). Crystalline bodies were not observed. RLOs observed in the midgut had evaginations of the cell wall membrane. These evaginations formed stalked knobs or "pili" that were approximately 24 nm in diameter with stalk lengths up to 56 nm (Figs. 4 and 7). The pili were randomly scattered over the outer surface of the RLO's cell wall. Extensive lysis of the gut cell cytoplasm was shown by the disruption of endoplasmic reticulum, Golgi complex, and mitochondria. Spherical VLPs of approximately 24 nm were observed aligned in groups in the degenerating fat body as well as partially enveloped particles 36 nm in diameter. These VLPs, which were similar to those observed in the pecan weevil diseased tissues, were also found in the fat body.

Mexican bean beetles. Abnormal larvae were lethargic and had necrotic spots on the hypodermis. Electron-microscopic examinations revealed RLOs in midgut, trachea, muscle, and hypodermal tissues. Figure 5 shows extensive damage to the midgut microvilli caused by RLO invasion. The only forms of the RLO observed in the specimens were secondary cells measuring approximately 0.5 μm × lengths up to 2.4 μm (Fig. 5). The envelopes of the RLO secondary cells were modified with stalked knobs or pili similar to those observed in the RLO-infected squash beetles.

Spherical VLPs were found in many vesicles in the sheath of the muscle and tracheal matrix associated with the hypodermis (Figs. 6 and 8). These VLPs measured approximately 39–42 μm in diameter. In another specimen, developing VLPs of approximately 50 nm were found in viroplasm within the cytoplasm of posterior midgut cells (Fig. 9).

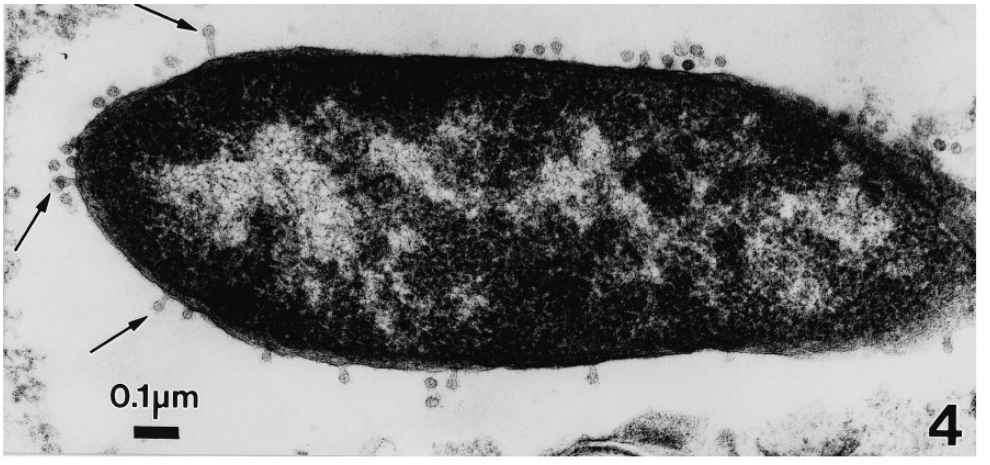
DISCUSSION

The most common microorganisms observed in the beetles were RLOs, which are similar to those included in the genus *Rickettsiella* (Weiss and Molder, 1984). A brief report of our initial examinations was made by Sikorowski (1985). The RLO "standard cells" and the "secondary cells" which we observed (by electron microscopy) infecting these three species of beetles were similar to the microorganisms found in the Japanese beetle (Adams, unpublished observation), *M. melolontha* (Devauchelle *et al.*, 1972), and *Paramyelois transitella* (Kellen *et al.*, 1972), with the exception that crystals were not observed in the vesicles of RLOs. Perhaps our samples were taken at an earlier stage of infection before crystal formation occurred or the RLOs

FIG. 1. Section of hypodermis of pecan weevil *C. caryae* showing *Rickettsiella*-like organisms (RLOs) (arrow) trapped in the cuticle during molting.

FIG. 2. Section of Malpighian tubule of pecan weevil larva infected with two pleomorphic forms of RLOs. Note the smaller condensed kidney-shaped bodies = elementary bodies = "standard cells" (points), coccoid bodies (small arrows), and the less dense, larger, bacteria-like forms = secondary cells (large arrows).

FIG. 3. Section of midgut of a squash beetle larva, *E. borealis*, infected with "standard cells" (points) and "secondary cells" (arrows). Note the dividing "secondary cell" (thick arrow).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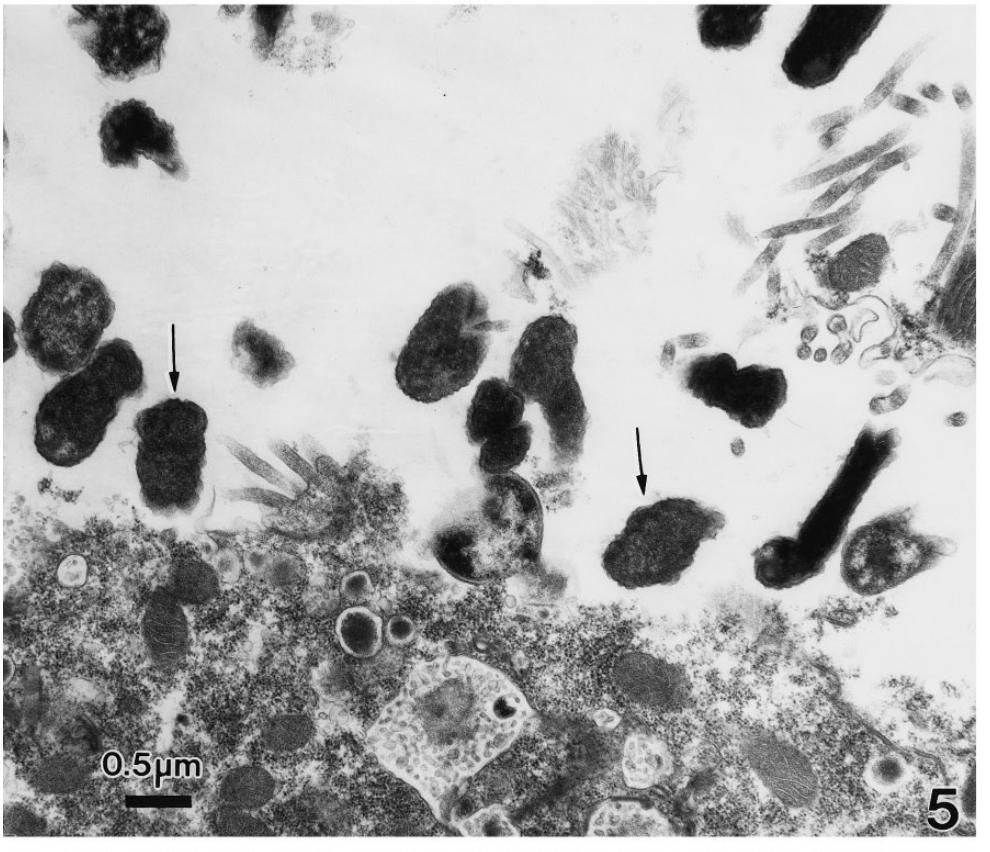
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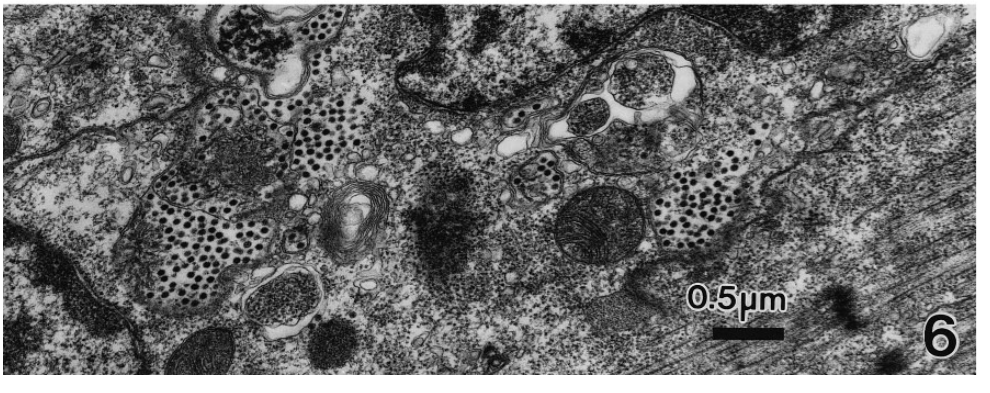
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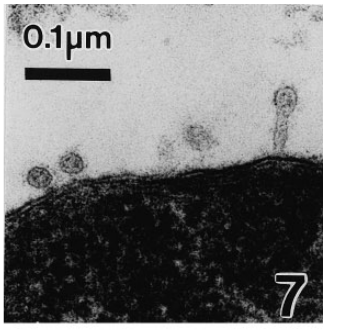
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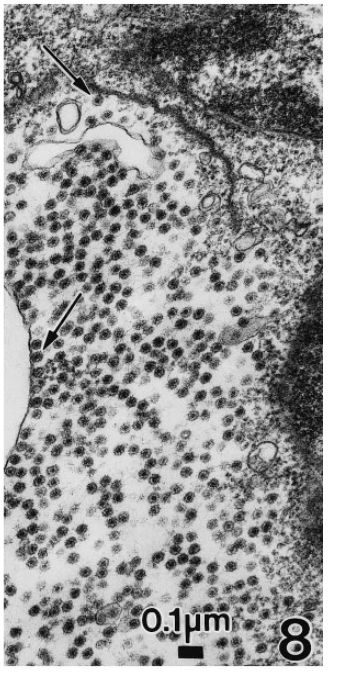
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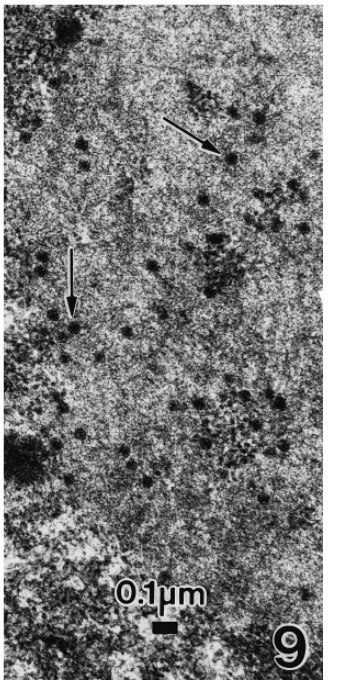
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described above do not form crystals in their developmental cycle. Unfortunately, projected serological studies to compare these RLOs with *R. popilliae* were never pursued because of the untimely death of Dr. T. B. Clark. The stalked knobs or pili observed on the cell wall membranes of the RLOs in the squash beetles and Mexican bean beetles are similar to those described in a species of Lepidoptera by Entwistle *et al.* (1968).

The susceptible tissues of the three species examined, which contained the VLPs described above, showed less ultrastructural degradation than the tissues which were infected with the RLOs. It was not possible to isolate the VLPs to test their virulence, but infections in midgut, muscle, and tracheal matrix can greatly interfere with the insect's normal physiological processes. Although biochemical tests will be required to precisely classify them, the VLPs observed in this study of 40 nm in diameter fall within the size range of the Tetraviridae (Reinganum, 1991; Murphy *et al.*, 1995). The VLPs of approximately 18-nm diameter may be similar to those reported in the subfamily Densovirinae (Murphy *et al.*, 1995). The VLPs of 50 nm are possibly immature stages of reoviruses that range from 56 to 80 nm in diameter (Hukahara and Bonami, 1991), are considerably smaller in diameter than those reported by Kitajima *et al.* (1985), and also differ morphologically from those found earlier in other specimens of Mexican bean beetles (Adams *et al.*, 1979).

Reports on rickettsia as the most common pathogen and a nonoccluded virus as an additional pathogen include Krieg (1962a), Moore *et al.* (1974), Louis *et al.* (1986), Degrugillier *et al.* (1991), and Shaw and Moloo (1993).

Reviews on rickettsial diseases of insects include those by Krieg (1962b, 1987), Weiss and Moulder (1984), Jackson and Glare (1992), and Tanada and Kaya (1993).

Some investigators have been apprehensive about using rickettsia for microbial control of insects. Recent studies indicate that *Rickettsiella* sp. are strictly insect pathogens and are not closely related to the rickettsia and chlamydia that are pathogenic to humans and animals (Frutos *et al.*, 1989, 1994). Diagnosis of "sick" insects to identify the microorganisms that cause problems in insect rearings and in the field is still a vital link in the development of better strategies for microbial control of insect pests. A knowledge of symptoms of diseases and possible corrective actions will greatly help those who are involved in rearing "disease-free" insects for biological studies.

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FIG. 4. Section of RLO "secondary cell" in a squash beetle larva. Note the stalked knobs or "pili" (arrows) extending from the outer surface of the outer membrane.

FIG. 5. Section of the midgut of a Mexican bean beetle, *E. varivestis*, larva showing the destruction to the microvilli of the columnar cells by the invading RLOs (arrows).

FIG. 6. Section of muscle and trachea associated with the hypodermis of a Mexican bean beetle larva showing vesicles containing spherical VLPs of 39–42 nm in diameter.

FIG. 7. Higher magnification of "pili" on the envelope of the RLO found in the squash beetle.

FIG. 8. Enlargement of VLPs found in vesicles near muscle and tracheal matrix tissues of Mexican bean beetle larvae (arrows).

FIG. 9. Viroplasm containing developing VLPs of approximately 50 nm in diameter (arrows) which were observed in the cytoplasm of the posterior midgut of Mexican bean beetle larvae.

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