COXIELLA POPILLIAE, N. SP., A RICKETTSIA CAUSING BLUE DISEASE OF JAPANESE BEETLE LARVAE

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Blue disease, a fatal infection of larvae of the Japanese beetle, *Popillia japonica*, was first noted in larvae collected by P. S. Clarke at Nottingham, Pennsylvania, in October 1940. It has since been encountered in larvae from several other widely separated points. At some of these localities only a few infected larvae were found, whereas at others fairly large numbers of diseased individuals were found repeatedly in surveys of the areas. While most of the recoveries of the disease have been in Japanese beetle larvae, the disease has also been diagnosed in larvae of two other scarabaeid species, *Phyllophaga anxia* and *P. ephilida*. This would suggest that still other insect species may be affected.

The disease has been recovered in only a small proportion of the sites examined by field surveys, but in areas where the disease was found as many as 17 out of 50 larvae recovered in individual diggings were affected. Therefore, blue disease may have potentialities as an important insect control agency. This paper presents a summary of the studies that have been made to date at the Moorestown and Beltsville laboratories of the Bureau of Entomology and Plant Quarantine to characterize the disease and its causal organism and to serve as the groundwork for further laboratory and field studies.

DESCRIPTION OF THE DISEASE

Gross characteristics. Larvae affected by blue disease are characterized by a greenish-blue discoloration of the fat body, which is discernible by careful gross external examination. The discoloration of the fat body is the only outward manifestation of disease that has been observed in the early stages of the disease. The activity of affected larvae may remain normal for more than a month after the first macroscopic evidence of infection has been noted. In experimental infection this occurred about 3 weeks after inoculation. The time required for the development of external symptoms was dependent on the temperature of incubation and the inoculating dosage employed. As the disease progresses, the discoloration of the fat body increases and becomes more readily apparent, but in most cases of experimental infection this was considerably less than that found in field infection, due possibly to the higher temperatures employed in the laboratory studies. Shortly before death the larvae become sluggish in their motions and cease feeding. They may then assume the somewhat milky appearance that characterizes the terminal stage of the disease in field collected specimens, which is due to the increase in the turbidity of the blood. After death

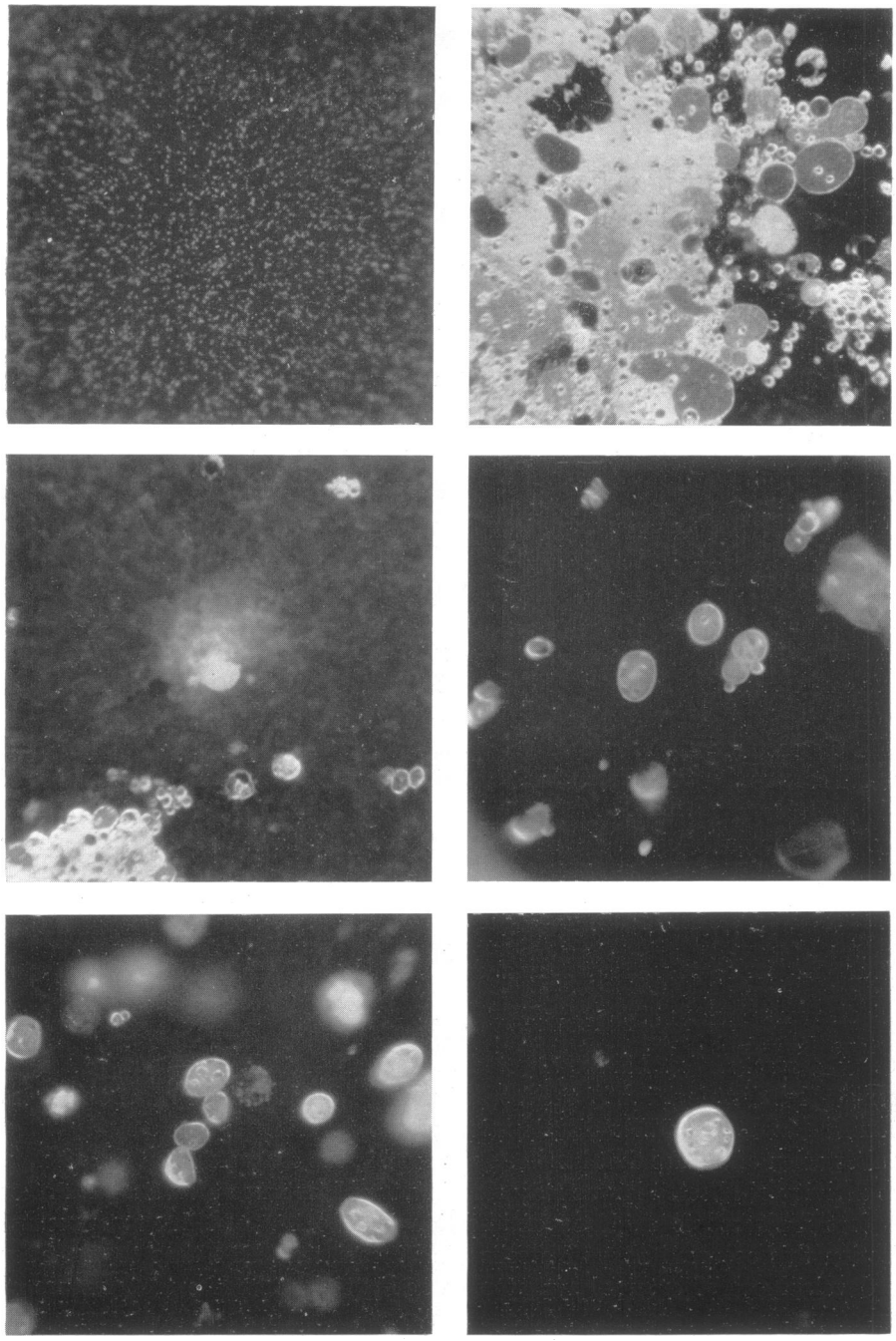

Figure 1. (upper left) Dark field photomicrograph of blue disease organisms, dried on slide and mounted in methyl alcohol. Magnification about 510 ×.

Figure 2. (upper right) Dark field photomicrograph of unfixed blood from blue-diseased larva. Note crystals and masses of organism (dense white areas) liberated from ruptured cells. Magnification about 510 ×.

Figure 3. (center left) Dark field photomicrograph of blood showing ruptured infected cell liberating a cloud of the blue disease organism. Magnification about 510 ×.

Figure 4. (center right) Dark field photomicrograph of unfixed blood from blue-diseased larva. Note infected cell with single crystalline inclusion. Magnification about 510 ×.

Figure 5. (lower left) Dark field photomicrograph of unfixed blood from blue-diseased larva. Note infected cells with crystalline inclusion and characteristic doublets free in hemolymph. Magnification about 510 ×.

Figure 6. (lower right) Dark field photomicrograph of a single infected cell containing 12 crystalline inclusions. Magnification about 510 ×.

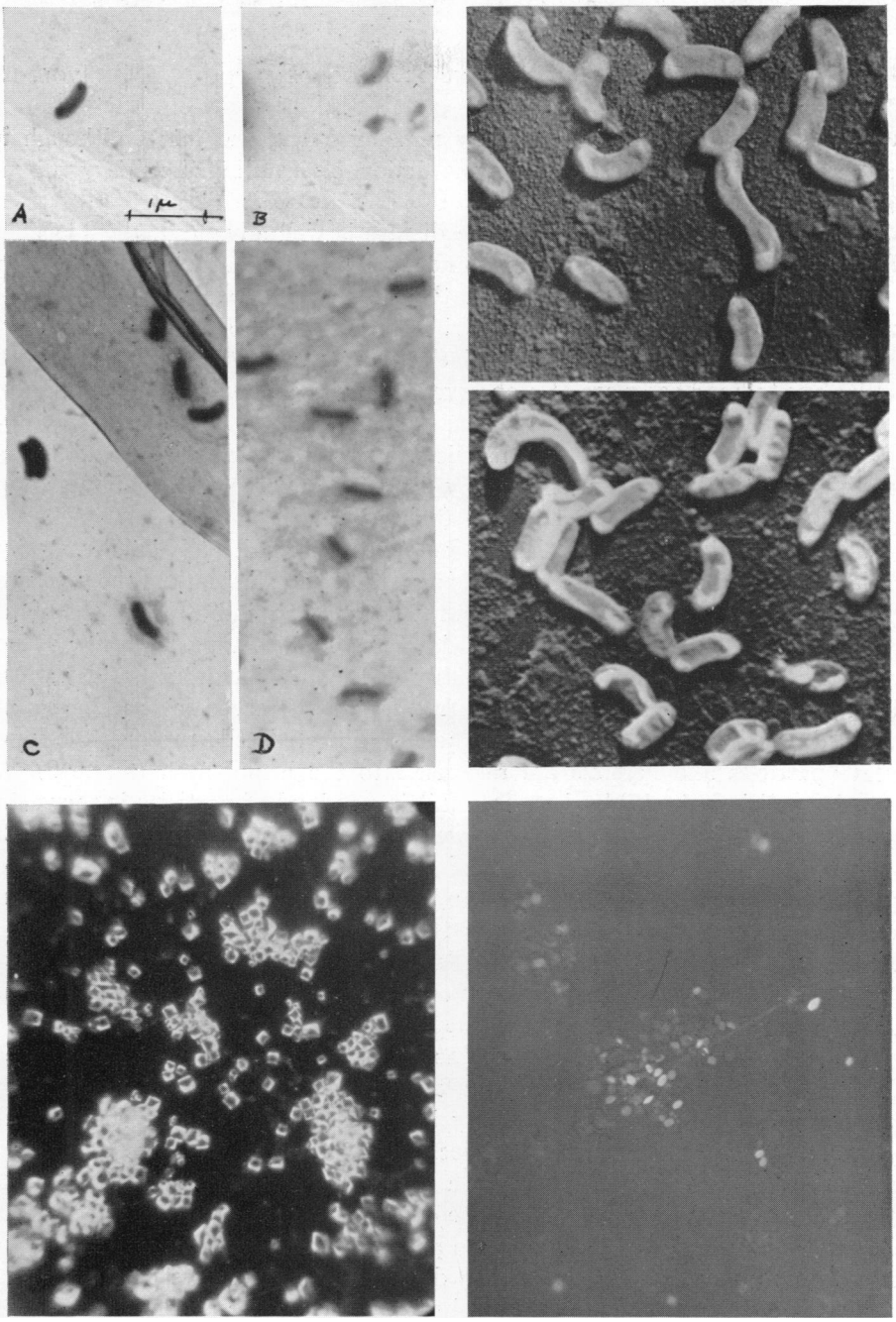

Figure 7. (upper left) Electron micrographs of the blue disease organism. Magnification about 10,000 ×.

A. Single cell exhibiting denser areas at the poles. From *Popillia japonica.*

B. Single cell outlined by capsule-like structure. From *Popillia japonica.*

C, D. Blue disease organisms from field infected *Phyllophaga ephilida* larva.

Figure 8. (upper right) Electron micrographs of palladium shadowed blue disease organisms. Magnification about 18,000 ×. (Prepared by K. M. Hughes and used with permission of E. A. Steinhaus.)

Figure 9. (lower left) Dark field photomicrograph of crystals from blue disease filtrates. Crystals dried on slide and mounted in ethyl alcohol. Magnification about 510 ×.

Figure 10. (lower right) Photomicrograph of crystals from blue disease filtrates under polarized light (crossed polaroids). Crystals dried on slide and mounted in anethole. Magnification about 510 ×.

the integument of the larvae generally remains strong and intact although it may be weakened or destroyed by the action of invading secondary microorganisms.

Microscopic characteristics. When the disease is well advanced, the blood of affected larvae contains enormous numbers of minute particles that exhibit pronounced Brownian movement and that are barely visible by bright field examination. Infected cells whose nuclei are packed with similar particles and contain one or more larger crystalline inclusions are also observed in the blood. Similar crystalline bodies are also observed free in the hemolymph. The minute particles were early considered to be the exciting agent of the disease, and subsequent studies have supported this view. These particles were found to be clearly visible and highly refringent by dark field microscopy, and their size order was estimated as 0.2–0.4 micron (figure 1).

Since the blood from normal larvae was found to be virtually free from any particles in this size order that are visible by dark field examination, the presence of the disease and its state of advancement were clearly indicated by the numbers of refringent particles found per field. Infected cells appear intensely blue by dark field examination and are readily distinguishable from healthy cells. The number of blue infected cells and free crystals observed in the blood also increased as the disease progressed. When the infected cells rupture, the blue disease particles and crystals are released into the hemolymph (figure 2). A rupturing cell in a fresh blood mount emitting a cloud of particles is shown in figure 3. The nuclei of infected cells were observed to contain from 1 to 12 crystalline inclusions (figures 4, 5, and 6), whereas the packed blue disease particles in these cells were estimated to number in the hundreds of thousands. Reasonably accurate counts of the blue disease particles were made with the Petroff-Hausser bacteria counter with dark field illumination. From counts on filtered suspensions of triturated experimentally infected larvae, it was calculated that at death there were 325 billion blue disease particles in a single larva.

No microscopic change in the blood has been observed during the early stages of the disease. When first macroscopic evidence of disease is noted and even after the discoloration of the fat body is pronounced, the microscopic blood picture is essentially normal. Only an occasional infected cell and very few blue disease particles free in the hemolymph can be observed. Examination of the fat body at this time shows the presence of large numbers of bluish infected fat cells containing myriads of blue disease particles and crystalline nuclear inclusions. These observations suggest that during the early stages of disease the infection is restricted largely to the fat body and that most of the infected cells observed in the blood during the later stages of disease originate from these loose-tissue aggregates.

DESCRIPTION OF THE CAUSAL ORGANISM

Morphology. As mentioned before, the refringent particles observed by dark field microscopy and referred to previously as "blue disease particles" were considered to be the causal organism or exciting agency of the disease. From

the dark field observations it was not possible to ascertain their size or shape, partly because of their rapid and erratic movement in fluid mounts. Their erratic movements and tendency to turn indicated that the particles were most probably elongate; they were thus considered to be minute rods. Furthermore, since the particles appeared uniformly bright in dark field and did not exhibit a dark center portion, it was concluded that they were below the limits of resolution, at least in respect to their shorter dimension (figure 1). Examination of suspensions of the particles deposited and dried on support films with the electron microscope (figures 7 and 8) showed the particles to be kidney-shaped rods 0.2 μ in width and 0.6 μ in length. These kidney-shaped rods were observed in all preparations studied and were the only objects noted in the size order estimated for the particles from dark field examination. Furthermore, the number of these kidney-shaped rods observed in the preparations was in good agreement with that calculated from the particle density of the suspension, the volume of suspension deposited, and the original area of deposition. Electron micrographs of the organism from an experimentally infected *Popillia* larva (figure 7, A and B) and from a field infected *Phyllophaga ephilida* larva (figure 7, C and D) show that the organism has the same morphology in both hosts. Further proof that the organisms from the two hosts were identical was obtained by infecting *Popillia japonica* larvae with suspensions prepared from field infected *Phyllophaga ephilida* larvae. Most of the rods observed were uniformly opaque to the electron beam, whereas others were nonuniform and suggested internal structure, some showing denser areas at the poles (figure 7, A), and others showing a denser longitudinal area surrounded by a lighter well defined capsule-like structure (figure 7, B). Part of the apparent nonuniformity of opacity may have been due to uneven collapse of the cells on drying as is indicated by the palladium-shadowed preparations (figure 8).

Filterability. Dark field counts on suspensions of the blue disease organism before and after filtration demonstrated that the organism passes quantitatively through coarse and medium porosity diatomaceous filters. In one such test an aqueous blood suspension before filtration contained 1.18 billion blue disease organisms per cm^3, whereas after filtration through a new sterile Mandler 7 lb test filter the density was determined to be 1.08 billion organisms per cm^3. In another test a suspension was first filtered through a 7 lb test filter and then passed through a new sterile Mandler 13 lb test filter. The density of the suspension before the second filtration was 55.2 billion organisms per cm^3, whereas after the second filtration the density was 33.0 billion per cm^3. Therefore, about 60 per cent of the organisms passing the medium porosity filter also passed the fine porosity filter.

DESCRIPTION OF THE CRYSTALLINE INCLUSIONS

The crystalline bodies present in the suspensions also passed through these filters. These bodies were mostly 1 to 3 μ in size and were centrifuged from the filtrates for study. Most of the crystals were bipyramids with a central prismatic section and appeared to belong to the tetragonal system. In basal view they

were isotropic and square in outline. In side view they were strongly birefringent and elongate hexagons in outline, the longest dimension corresponding to the diagonal of the square observed in the basal view (figures 9, 10). The inclusion bodies inside the infected cells or freshly extruded from these cells did not appear as sharply angular as the crystals obtained from the filtrates and generally occurred as doublets, a larger and a smaller body joined together. These doublets were also observed in the filtrates but in smaller number than the individual crystals. It has not been fully established whether or not the sharply angular crystals observed free in the blood and in filtrates are identical with the less

TABLE 1

Effect of dosage on blue disease development at 23.9 C. Fifty larvae used in each test

NUMBER OF ORGANISMS INJECTED	NUMBER OF LARVAE DEVELOPING SYMPTOMS	MEDIAN TIME FOR DEVELOPMENT OF DISEASE SYMPTOMS	MEDIAN ADJUSTED DEATH TIME DUE TO DISEASE
		days	*days*
0	0	—	—
970	44	24	48
13,000	46	23	49
154,000	42	22	43
1,800,000	48	21	42
22,500,000	47	20	39
253,000,000	9	18	18

TABLE 2

Effect of temperature on blue disease development

TEMPERATURE OF INCUBATION	NUMBER OF LARVAE IN TEST	NUMBER OF LARVAE DEVELOPING SYMPTOMS	MEDIAN TIME FOR DEVELOPMENT OF DISEASE SYMPTOMS	MEDIAN ADJUSTED DEATH TIME DUE TO DISEASE
			days	*days*
26.7 C	200	176	19	44
23.9 C	100	81	23	46
21.1 C	100	85	24	52

angular bodies observed within the nuclei of infected cells, but further study should clarify this point.

EXPERIMENTAL TRANSMISSION OF THE DISEASE

By injection. It was found that healthy Japanese beetle larvae could be infected by injecting them with suspensions of diseased blood or with filtrates of these suspensions. Since attempts to isolate and culture the causal organism on artificial media were unsuccessful, material for study could be maintained only by serial inoculation of insect larvae. Initially only fresh blood suspensions were employed. Later tests showed that suspensions were still infectious after 76 days' refrigeration at 4 C. The use of refrigerated suspensions greatly simplified the work of maintaining the disease for study.

The microinjector developed at the Moorestown laboratory for use in the

milky disease work (Dutky and Fest, 1942) was employed in these studies. Dosages were determined and standardized by dark field counts on the suspensions used as inocula. The injected larvae were incubated at constant temperature in individual tins of soil with sprouted seed added as food. Gross examination of the larvae for the development of external symptoms was made at short intervals. This was done by examining the larvae under a strong beam of light, noting especially the thin layer of the fat body overlaying the small intestine and the dark rectal sac. It is here that the bluish fluorescence of the diseased fat body is earliest detected.

Preliminary studies indicated that 30 C is very nearly the upper temperature limit for disease development. At this temperature, development of external symptoms was not consistent, nor were the symptoms as marked as at lower temperatures. In these tests 26.7 C was found to be optimum for disease development. The lower limit was not determined. Data from representative tests showing the effect of dosage and of temperature on disease development are given in tables 1 and 2.

By soil inoculation. Healthy larvae were also infected by holding them in soil inoculated with suspensions of the organism. In this test, 6 positive cases of infection and 4 doubtful cases were diagnosed among 50 larvae exposed for 40 days to soil inoculated with 720 billion organisms per kilogram of soil.

DISCUSSION

The taxonomic position of the blue disease organism is not clear. The shape of the organism is strongly suggestive of *Vibrio* whereas its small size and intracellular growth seem to rule out this genus as a logical classification. Many of its characteristics are similar to those described for the polyhedral viruses attacking lepidopterous insects (Steinhaus, 1949). It is, however, somewhat larger than those described as the causal organisms of recognized polyhedral virus diseases. The authors have had the opportunity to submit material to Dr. E. A. Steinhaus of the Laboratory of Insect Pathology, University of California, for his examination and study. Dr. Steinhaus considers the organism to resemble more nearly a rickettsia than a virus of any of the types now known to occur in insects. He suggests that it might safely be placed in the order *Rickettsiales* as a new genus of the family *Rickettsiaceae* or that it might represent an entirely new family. In agreement with these suggestions and in view of the filterability of the organism, the authors have selected the name *Coxiella popilliae*, n. sp., as being most nearly descriptive of the blue disease organism.

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SUMMARY

Blue disease, a fatal infection of Japanese beetle larvae, is characterized by a greenish-blue discoloration of the fat body. The causal organism is a minute, filterable, kidney-shaped rod 0.2 μ in width and 0.6 μ in length which develops largely in the nuclei of infected cells. The blue disease organism has been named *Coxiella popilliae*.

REFERENCES

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