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COMPARATIVE HISTOPATHOLOGY OF INFECTION BY *OVAVESICULA POPILLIAE* [*MICROSPORIDA : PLEISTOPHORIDAE*] IN LARVAL AND ADULT JAPANESE BEETLES, *POPILLIA JAPONICA*

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The host response to infection and tissue susceptibility of larval and adult Japanese beetles, *Popillia japonica* Newman, to the microsporidium, *Ovavesicula popilliae* Andreadis & Hanula, are reported. The normally transparent Malpighian tubules of Japanese beetle larvae, were hypertrophied and white in color when infected with *O. popilliae*, a microsporidian which also infects larval fat body, epidermis and pericardial cells. In addition to these tissues, œnocytes and tracheal epithelial cells were also infected in adults. Adult and larval reactions to infection included hypertrophied cells and melanization of the pericardium, but only larvae exhibited an intense inflammatory response. The discoloration of the pericardium most likely resulted from an accumulation of melanin.

KEY-WORDS : *Ovavesicula popilliae*, microsporida, *Popillia japonica*, histopathology, gross pathology, inflammatory response.

Ovavesicula popilliae Andreadis & Hanula is a recently discovered microsporidium of the Japanese bettle, *Popillia japonica* Newman (**Andreadis & Hanula,** 1987). It is widely distributed in Connecticut (**Hanula & Andreadis,** 1988), is transmitted horizontally, and appears to produce a chronic, debilitative disease. In our initial description, we found that the parasite developed primarily in the Malpighian tubules of larvae. However, further investigations revealed more generalized infections in transtadially infected adults and, on occasion, systemic infections in larvae. In addition, we have found heavily infected larvae with melanized nodules that are rare in adults, suggesting that the 2 stages differ in their response to the microsporidium. In this study, the histopathology of *O. popilliae* in larvae and adults was examined to determine tissue specificity, and differences in host reaction to *O. popilliae* invasion. Detailed observations on gross pathology are also included to facilitate diagnosis of infection.

METHODS

GROSS PATHOLOGY

Observations of gross pathology were made on approximately 2 000 larvae collected over a 2 year period from locations throughout Connecticut and on 500 adults collected

from one location during a 10 week period. Individuals with various degrees of infection were dissected and examined for signs of infection. The Malpighian tubules were then removed, smeared on slides, stained with a 20 % Geimsa solution, and examined microscopically (1 000 ×) to confirm infection and determine the accuracy of our initial observations.

Histopathology

Naturally infected beetles were collected from a population in Norwalk, Connecticut previously determined to have a high prevalence of *O. popilliae* (**Hanula & Andreadis,** 1988). Larval histopathology was based on 5 third instars that were starved for 48 h following collection (to clear their guts of soil particles), and then whole larvae were fixed in Carnoy's solution for 3 h. Following fixation, the head and legs were excised, and the larvae were cut in half. Specimens were then dehydrated through an ethanol and butanol series, and embedded in Paraplast™ Plus. Serial longitudinal sections were cut at 6-7 μm, stained with Heidenhain's hematoxylin and Eosin Y, and examined microscopically.

Five infected adults were processed in a similar manner, except they were embedded whole and examined in cross-section.

RESULTS

Gross pathology

No characteristic external symptoms of infection were found in either stage, but microscopic examination of feces for sporophorous vesicles was an effective means of non-destructively diagnosing moderate to heavy infections in adults as well as larvae.

Malpighian tubules from healthy larvae were transparent or slightly yellow, smooth, and about the same diameter throughout (fig. 1). In contrast, infected tubules were typically swollen, entirely white or mottled, and frequently had melanized nodules adhering to them (fig. 1). The white discoloration of Malpighian tubules in larvae was always associated with the presence of spores, even in lightly infected tubules that were not noticeably distended. However, no abnormalities in color or shape could be detected in lightly infected tubules containing only vegetative stages.

The anterior portions of healthy adult Malpighian tubules were similar to those observed in larvae. However, the posterior portions were usually white due to the accumulation of uric acid crystals ; thus white discoloration could not be used as a pathognomonic sign of infection in adults. The presence of hypertrophied tissue was the only gross indication of infection in adults. In addition, infected adults rarely contained melanized nodules which were common in larvae.

Approximately 90 % (n = 300) of *O. popilliae* infections in larvae, and 95 % (n = 500) in adults, were accurately diagnosed during dissection using the above criteria.

Histopathology

The examination of heavily infected larvae histologically revealed a number of infected tissues in addition to Malpighian tubules. These included, fat body cells in close proximity to infected tubules (fig. 2), and a limited number of epidermal (fig. 3) and pericardial cells (fig. 4). Although extensive infections were observed in these tissues, only lightly infected cells are shown for comparison to uninfected cells. We did not find *O. popilliae* in muscle, gut or other tissues.

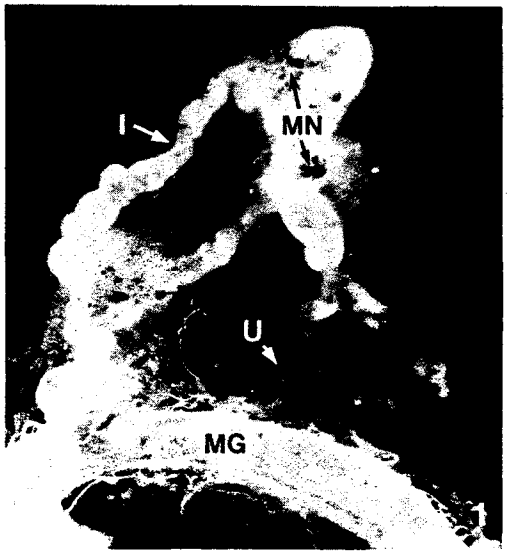

Fig. 1. The midgut (MG) and Malpighian tubules of a Japanese beetle larva infected with *Ovavesicula popilliae* showing the extensive swelling and white discoloration of infected tubules (I) and the melanized nodules (MN) associated with infection. U, uninfected tubules. × 26.

Larval reaction to *O. popilliae* was characterized by hypertrophication of infected cells, an intense inflammatory response, and melanization of the pericardium. Hypertrophied cells containing enlarged nuclei (fig. 5) were common in a number of tissues including the Malpighian tubules. The inflammatory response resulted in large aggregations of hemocytes near infected tissue, often with melanized cores (fig. 6). These melanized nodules contained large numbers of sporophorous vesicles. Melanization of the pericardium was evident in the 3 heavily infected specimens. Normal pericardial cells contained numerous vacuoles that stained red with Eosin Y (fig. 4), but in heavily infected larvae brown or black granules were found throughout the pericardium (fig. 7). The intensity of the melanization appeared to coincide with the degree of infection and the intensity of the inflammatory response. However, melanized pericardial cells were generally not infected. Melanization of the pericardium was evident during dissections and was occasionally visible in living larvae as 2 dorsolateral stripes beneath the cuticle. However, this was only detected in very heavily infected individuals. We also observed melanized pericardial cells in 1 larva infected only with milky disease, *Bacillus popilliae* Dutky, and in another larva that had a large melanized area surrounding a wound. The latter was not infected by either pathogen.

In adults, we observed extensive infections in the Malpighian tubules and in the epithelium of air-sacs and tracheae (fig. 8). More infected pericardial cells were observed in adults than in larvae, and adult œnocytes (fig. 9) were also infected. We found no indication of *O. popilliae* in the reproductive organs of either sex, even though we observed extensive infections in tissues adjacent to these organs. Hypertrophied cells and melanization in the pericardial cells appeared to be common reactions to parasite invasion in both stages, although the adults did not exhibit the degree of melanization observed in some larvae. Hemocyte aggregations and melanized nodules were rarely observed in adults. However, in

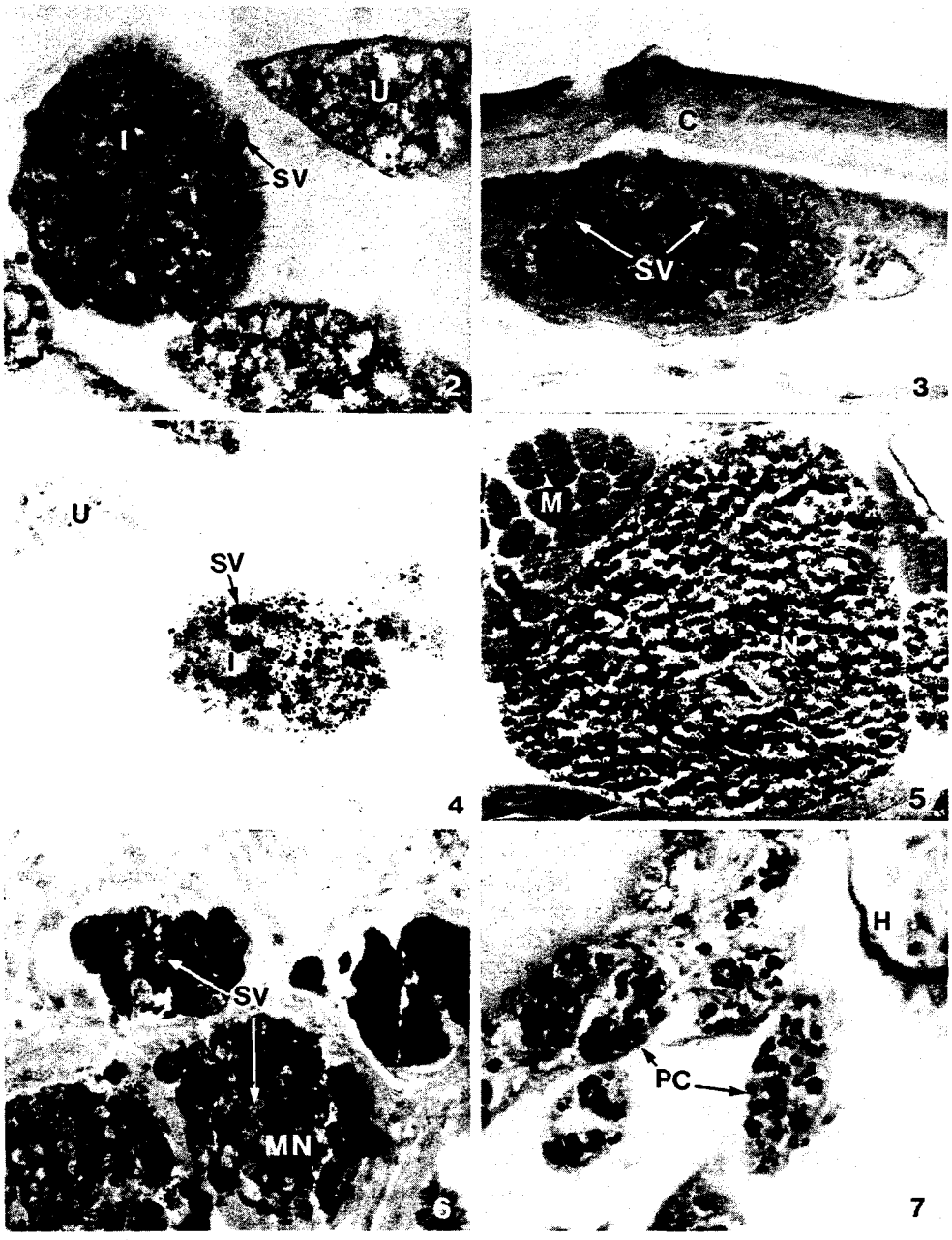

Fig. 2-7. Histological sections of tissues infected with *Ovavesicula popilliae* from larval Japanese beetles. (2) Fat body (× 270). (3) Epidermal cells (EC) (× 330). (4) Pericardial cells (× 220). (5) Hypertrophied cell filled with sporophorous vesicles (SV) (× 220). (6) Inflammatory response showing a hemocyte aggregation and melanized nodules (MN) containing sporophorous vesicles (× 230). (7) Melanized pericardial cells (PC) (× 220). C, cuticle ; H, heart ; I, infected cells ; M, muscle ; N, host cell nucleus ; U, uninfected cells.

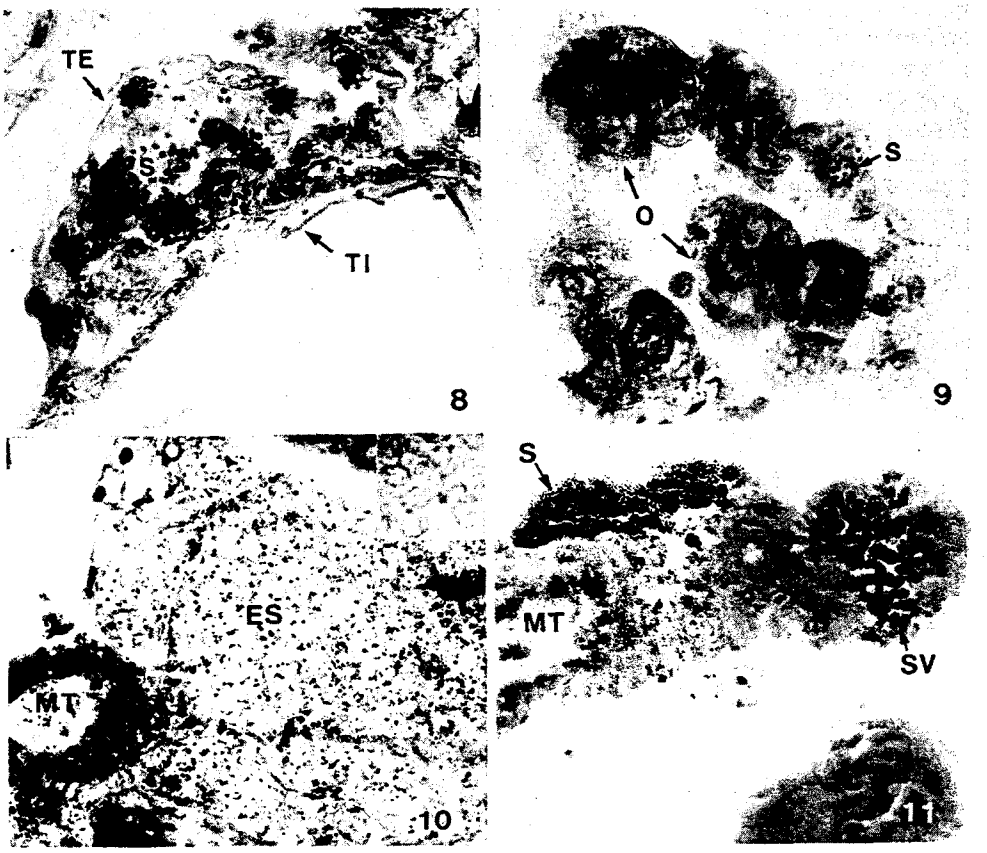

Fig. 8-11. Histological sections of tissues infected with *Ovavesicula popilliae* from adult Japanese beetles. (8) tracheal epithelium (TE) (× 625). (9) Œnocytes (O) (× 545). (10) Unidentified tissue containing large numbers of empty spores (ES) (× 210). Inset, empty spores (× 1 500). (11) Malpighian tubules (MT) shown associated with free spores (S) and sporophorous vesicles (SV) (× 215). T, tracheal intima.

several individuals we observed extensive areas of infected tissue that contained mostly empty spores (fig. 10). This tissue was not recognizable but it was generally associated with the Malpighian tubules and gut.

Occasionally, adults and larvae contained heavily infected cells filled with free spores not enclosed in sporophorous vesicles (fig. 11). The cells involved were generally unrecognizable and were located throughout the hemocoel. The reason for the breakdown of the sporophorous vesicles in these tissues or cells is unknown.

DISCUSSION

The Malpighian tubules of Japanese beetle larvae were hypertrophied and white in color when infected with *O. popilliae*. Larval fat body, epidermis and pericardial cells were suitable for *O. popilliae* development as well. In addition to the tissues infected in larvae,

adult œnocytes and tracheal epithelial cells were also susceptible. Adult and larval reactions to infection included hypertrophied cells and melanization of the pericardium, but only larvae exhibited an intense inflammatory response.

Melanin accumulation in the pericardium has not been previously reported. The hemolymph of heavily infected larvae immediately turns black when exposed to air. This reaction does not occur in the hemolymph of healthy individuals, but it has been reported for larvae infected with *Bacillus popilliae* (**Beard,** 1945). **Mandrell** (1971) suggested that the pericardial cells take up waste or foreign proteins from the hemolymph, and **Chapman** (1971) indicated that melanin may accumulate in some cells as a form of storage excretion. **Crossley** (1972) demonstrated that colloidal or soluable molecules of less than 120 A entered pericardial cells but only selected molecules were sequestered and digested. Recently, **Fife** *et al.* (1987) demonstrated that pericardial cells were involved in synthesis and secretion of polypeptides, but it is unknown whether or not they are involved in melanin production. In Japanese beetles, excess melanin apparently accumulated in the pericardial cells. Whether the melanin was formed within the pericardium as a defensive reaction or was sequestered as a means of storage is unknown.

Kharazi-Pakdel (1968) studied the histopathology associated with *Nosema melolontha* Krieg in larvae of the scarab beetle *Melolontha melolontha* L., and found a limited inflammatory response and no hypertrophication. In contrast, we observed responses in Japanese beetles that were similar to those reported for microsporidia infections in several species of Lepidoptera (**Brooks,** 1971a,b ; **Kellen & Lindegren,** 1973).

The degree of inflammation we observed in Japanese beetle larvae may be an indication that *P. japonica* is not the natural host of *O. popilliae* or that this may represent a recent host-parasite association. A number of studies have suggested this for other host-parasite relationships (**Timberlake,** 1912 ; **Salt,** 1963 ; **Brooks,** 1971a). The host origin of *O. popilliae* remains to be determined, but it has not been observed in other closely associated Scarabaeid larvae in Connecticut (**Hanula & Andreadis,** 1988).

Brooks (1971) has suggested that an inflammatory response may be of little value in restricting the spread of microsporidia to other tissues, since he found *Nosema shpingidis* Brooks developing within the hemocyte nodules of *Manduca sexta* (L.) larvae. However, our observations suggest that the inflammatory response may be of some value in restricting the spread of *O. popilliae* to other tissues in *P. japonica* larvae, even though *O. popilliae* also developed in hemocyte nodules. We base this conclusion on our observations of a greater intensity of *O. popilliae* infection in secondary tissues outside of the Malpighian tubules in adult *P. japonica,* which exhibited little inflammatory response. Larvae exhibited intense inflammation and less infection in secondary tissues. However, the more widespread distribution in adults may have resulted from release of *O. popilliae* into the hemocoel due to host cell lysis during the pupal stage. No other comparative histopathology studies of microsporidium infections have been conducted, so we are uncertain whether this occurs in other insects.

We observed extensive areas containing empty spores in adults but not in larvae. In addition, the hemocyte capsules and melanized nodules that were common in larvae were not observed in adults, even though the adults were infected transtadially. The large areas of empty spores in adults were most likely the remains of hemocyte capsules formed in the larval stage. The melanin may have been reabsorbed during pupation, since large melanized nodules were not observed in over 500 adults we dissected. **Salt** (1970) found that capsules undergo modifications and reductions in size following formation. **Vey & Gotz** (1986) reported that some capsules in *Galleria mellonella* L. were enveloped by hypodermal cells, separated from the hemocoel and eventually eliminated at larval ecdysis.

Conversely, **Lackie** (1988) suggested that hemocyte capsules remain in the hemocoel until the insect dies. The fate of hemocyte capsules in Japanese beetles remains to be determined. However, our observations suggest that, while they appear to remain in the hemocoel during the pupal and adult stages, they apparently undergo extensive modifications.

Most of the information on hemocytic defense is based on bacteria, metozoan parasites or foreign objects placed in insect hemolymph *in vivo* and *in vitro* (**Dunn,** 1986 ; **Lackie,** 1988 ; **Nappi,** 1975 ; **Whitcomb** *et al.,* 1974). The hemocytic response of insects to microsporidia, which are obligate intracellular parasites, has received little attention. **Weiser** (1963) suggested that hemocytes are only active in microsporidian infections when the sporoplasm enters the hemocoel from the gut lumen, and in advanced infections after the host cells lyse and release parasites into the hemolymph. Our observation suggest that hemocytes aggregate near infected cells but we were uncertain if cell lysis had occurred. However, it is clear that hemocytes encapsulated host tissue along with the parasites. Further evidence of this can be seen in the light micrographs of **Brooks** (1971a) and **Kellen & Lindgren** (1973). In particular the hemocytic infiltration of fat tissue in response to a microsporidian infection (**Kellen & Lindgren,** 1973) suggests that hemocytes respond to factors other than contact with foreign objects as proposed by **Salt** (1970). In the case of microsporidian invasion, hemocytes may respond to an "injury factor" (**Harvey & Williams,** 1961 ; **Cherbas,** 1973) produced by the invaded cells or changes in the basement membrane surrounding the cells (**Lackie,** 1988 ; **Gunnarsson,** 1988). The study of microsporidia infected cells may provide additional information about how hemocytes recognize foreignness. Investigations of microsporidia that infect both a habitual host where no host defense is illicited and non-habitual hosts with strong hemocytic responses would be valuable.

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RÉSUMÉ

Histopathologie comparée de l'infection par *Ovavesicula popilliae* [*Microsporida : Pleistophoridae*] chez la larve et l'adulte de *Popillia japonica*

La réponse de l'hôte à l'infection et la sensibilité de la larve et de l'adulte de *Popillia japonica* Newman à *Ovavesicula popilliae* Andreadis & Hanula, sont rapportées. Les tubes de Malpighi des larves du Scarabé japonais normalement transparents sont hypertrophiés, de couleur blanchâtre quand ils sont infectés par les microsporidies qui infectent aussi le corps gras, les cellules épidermiques et péricardiales. En plus, les œnocytes et les cellules épithéliales de la trachée ont été aussi infectées chez l'adulte. Des cellules hypertrophiées et la mélanisation du péricarde caractérisent les réactions à l'infection des adultes et des larves, mais seules ces dernières manifestent une intense réaction inflammatoire. La coloration du péricarde la plus probable résultait d'une accumulation de mélanine.

MOTS CLÉS : *Ovavesicula popilliae,* Microsporida, *Popillia japonica,* histopathologie, pathologie globale, réaction inflammatoire.

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