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Brief Communication

Spherites in the midgut epithelial cells of the sugarcane borer parasitized by *Cotesia flavipes*

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ABSTRACT: *Diatraea saccharalis,* the main pest of sugarcane, has been controlled by *Cotesia flavipes.* Very little is known about the effect of parasitism on the host organs, including the midgut. The Lepidoptera midgut epithelium is composed of columnar, goblet, regenerative, and endocrine cells. Spherites have been described in columnar and regenerative cells of several Lepidoptera species, and presented a lot of functional meaning. We identified spherites in the midgut epithelial cells of non-parasitized *D. saccharalis* larvae analyzed the effect of parasitism on spherite morphology and distribution along the length of the midgut. Midgut fragments of both non-parasitized and parasitized larvae were processed for transmission electron microscopy. All the midgut epithelial cells showed spherites, but they were not preferentially located in a particular part of the cells. Parasitized larvae had more spherites, mainly in the columnar cells, than non-parasitized larvae. This observation was associated with an ionic imbalance within the insect host. Spherites were more abundant in the anterior midgut region than in other regions, which suggests that this region is involved in ion transport by intracellular and/or paracellular route. The morphological variability of spherites in the cells of parasitized larvae was related to the developmental stages of these structures.

Introduction

Spherites are spherical cytoplasmatic granules whose mineral content assume the form of concentric lamination. These structures have been described in cells of different organs in invertebrates. In addition to the presence of spherites in the insect midgut (Wright and Newel, 1964; Cruz-Landim, 1971, 2000; Nopanitaya and Misch, 1974; Turbeck, 1974; Waku and Sumimoto, 1974; Sohal *et al.*, 1977; Humbert, 1978; Serrão and Cruz-Landim, 1996, 2000; Cruz-Landim and Serrão, 1997), these structures are also present in insect Malpighian tubules (Teigler and Arnott, 1972; Ryerse, 1979; Krüger *et al.*, 1987; Hazelton *et al*., 1988; Spring and Felgenhauer, 1996; Cruz-Landim, 2000; Hazelton *et al*., 2001), in the midgut glands of invertebrates (Ludwig and Alberti, 1988; Lipovsek *et al.*, 2002, 2004; Pigino *et al.*, 2006), and in the mantle tissue of bivalves (Vesk and Byrne, 1999).

The chemical composition of these structures varies in invertebrates. Turbeck (1974) verified that spherites represent a calcium reserve, which also possesses magnesium in their structure, for several Lepi-

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doptera species. Lipovsek *et al.* (2002) demonstrated that young spherites of bivalves are composed of calcium and phosphorus in electron-dense layers and silicon in electron-lucent layers. The authors show that the layers of spherites contain organic material, such as glycoprotein and proteoglycans, associated with the inorganic compounds cited.

Little is known about the origin of these structures in the cells of insects. For Hymenoptera, it was proposed that the organic material, a precursor of spherites, may be produced within the rough endoplasmic reticulum, and the inorganic material also may be sequestered by this organelle, which may lose ribosomes that create the membrane surrounding the crystalloid (Cruz-Landim, 2000).

The sugarcane borer, *Diatraea saccharalis* Fabricius, is the main sugarcane pest and also affects other crops such as sorghum, corn, and rice. This pestilential insect has been controlled by the release, in nature, of the parasitoid *Cotesia flavipes* (Hymenoptera: Braconidae). The midgut epithelium in Lepidoptera is principally made up of columnar, goblet, regenerative, and endocrine cells (Lehane and Billingsley, 1996). Many studies suggest that the distribution, morphology, and morphometry of these cells may be variable throughout the length of the midgut (Santos *et al.,* 1984; Pinheiro and Gregório, 2003; Pinheiro *et al.*, 2003). We studied the ultrastructure of the spherites in midgut epithelial cells of *D. saccharalis* larvae, both non-parasitized and parasitized by *C. flavipes*. We correlated these findings with the spatial distribution of cells along the midgut.

Material and Methods

The insects were reared on an artificial diet and maintained under controlled conditions (26 $\pm$ 1ºC; 70 $\pm$ 5% humidity, and 14 h photophase). *Cotesia flavipes* females were left to oviposit on the dorsal surface of the last instar *Diatraea saccharalis* larvae. Parasitized and non-parasitized larvae were sampled at six days after parasitism. The insects were dissected; the midgut was removed and fragmented in the anterior and posterior regions. The midgut fragments were fixed in 2.5% glutaraldehyde – 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.3) for 24 h, post-fixed in 1% osmium tetroxide in the same buffer for 2 h, dehydrated through a graded series of acetone, and embedded in Araldite7 resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined under a Philips CM100 transmission electron microscope.

Results

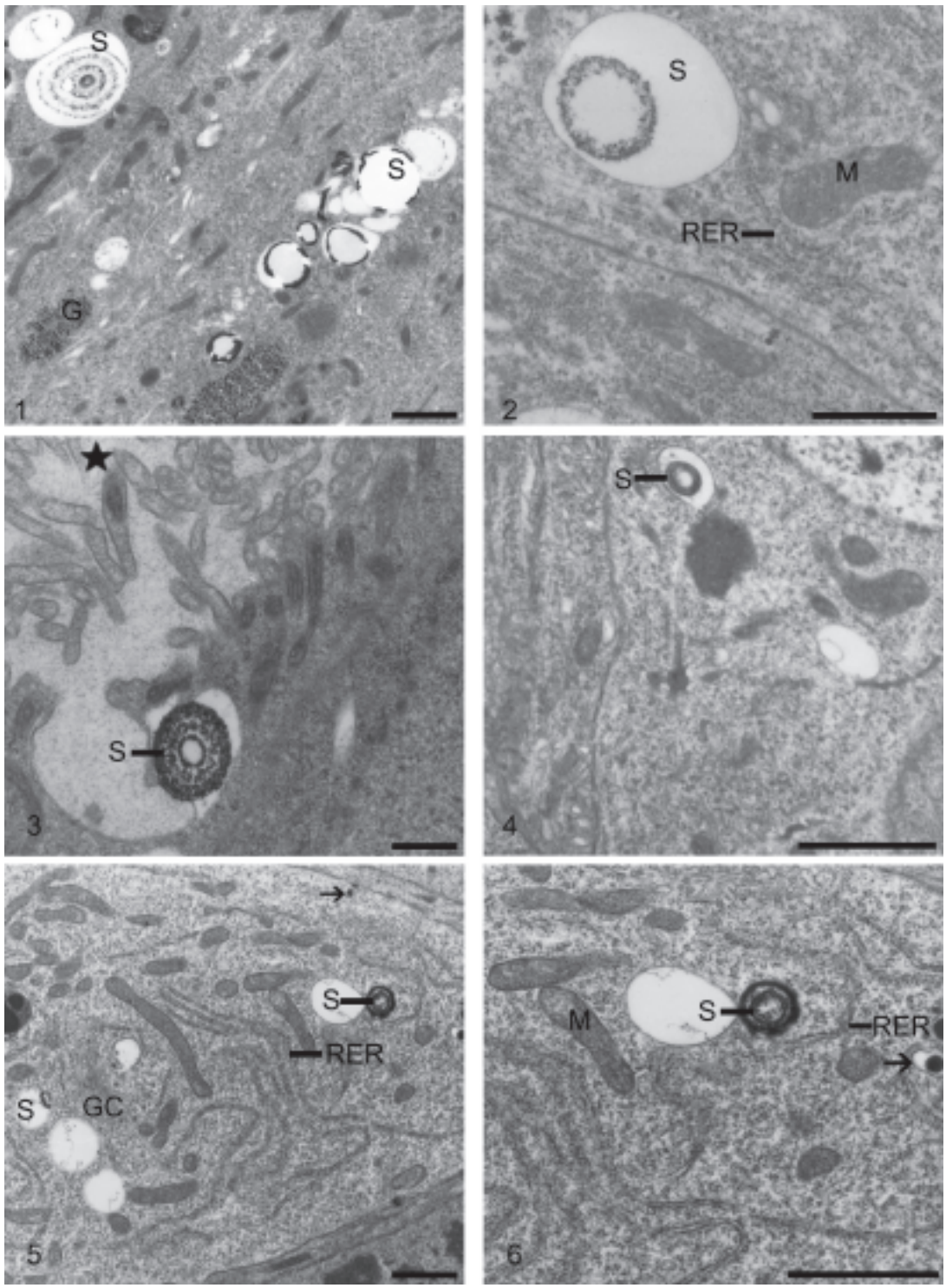
In non-parasitized *D. saccharalis* larvae, spherites were scarce in the cytoplasm of all epithelial cell types, in both the anterior and in the posterior midgut regions (Figs. 1-6). The spherites were not preferentially located within a particular part of the cell, and they varied in form, size, and structure. The spherites were electron-dense lamellas, which were located in the vacuolar membrane enwrapping, or which were filling the vacuole with several lamellas of variable thickness and concentric disposition (Figs. 1-6).

In parasitized larvae, the spherites were abundant and varied in size and form; they were present in the cytoplasm of all epithelial cell types, most frequently columnar cells, especially those of the anterior midgut region (Figs. 7-11). The spherites were voluminous and generally occurred in thinner lamellas than those observed in non-parasitized larvae (Figs. 7-13). The internal morphology was also variable. Some spherites did not occur in structured lamellas, and they contained finely flocculated material of low electron density that were concentrated close to the vacuolar membrane in some cases (Figs. 7, 9-10). Other spherites possessed only a thin lamella or innumerable concentric lamellas (Figs. 7-9, 11-13). The grouped or isolated spherites were not preferentially located within a particular part of the epithelial cells (Figs. 7-13). The spherite membranes were frequently fused (Figs. 8-11). But scarce spherites were noted in the posterior midgut region, and those observed were small and with few lamellas (Figs. 12-13). We did not observe the release of spherites into the midgut lumen in any epithelial cell from the two midgut regions.

Discussion

To date, spherites have been described only in columnar and regenerative cells of the insect midgut (Turbeck, 1974; Cruz-Landim, 1971). Therefore, an interesting finding of our study is the spheres, crystals, or spherites inside the cytoplasm of the four cell types in the midgut epithelium of *D. saccharalis,* both non-parasitized and parasitized by *C. flavipes*.

Independent of the type and location of midgut epithelial cells in *D. saccharalis,* the spherite morphology varies as much in relation to crystalloid inclusion ultrastructure as in relation to the size and form of the surrounding vacuole. Spherites have been described, by use of electron transmission microscopy, as membra-



FIGURES 1-6. Epithelial cells from the midgut of non-parasitized *Diatraea saccharalis* larvae: spherites (S); glycogen (G); rough endoplasmic reticulum (RER); goblet cell cavity (★); granules (arrow); mitochondria (M); Golgi complex (GC). **Fig. 1.** Columnar cell. Bar = 1μm. **Fig. 2.** Columnar cell. Bar = 0.5μm. **Fig. 3.** Goblet cell. Bar = 1μm. **Fig. 4.** Regenerative cell. Bar = 1μm. **Fig. 5.** Endocrine cell. Bar = 1μm. **Fig. 6.** Endocrine cell. Bar = 1μm.

nous vesicles containing layers of electron-dense material, interspersed with other electron-lucent content, which form concentric lamellas (Cruz-Landim, 1971, 2000; Nopanitaya and Misch, 1974; Turbeck, 1974; Serrão and Cruz-Landim, 1996; Corrêa *et al.*, 2002, 2003; Lipovsek *et al.*, 2002, 2004). In a study by Wright and Newell (1964), columnar cells from *Anystis sp* did not show with precision the spherite vacuolar membrane. This finding was probably the result of technical problems, considering that subsequent studies, after improvement in fixation techniques, clearly showed that the spherites are delimited by membrane (Nopanitaya and Misch, 1974; Turbeck, 1974; Serrão and Cruz-Landim, 1996; Cruz-Landim, 2000; Corrêa *et al.*, 2003).

The location of spherites in insect epithelial cells has been described as varied. Turbeck (1974) showed, in seven Lepidoptera species, that spherites were preferentially located in the apical cytoplasm in both columnar and regenerative cells. The same was described by Cruz-Landim (1971) for midgut columnar cells of *Trigona postica*. However, Serrão and Cruz-Landim (2000), in studying the midgut of Meliponinae larvae, observed spherites in the middle portion of columnar cells. In the midgut of *D. saccharalis*, in both non-parasitized and parasitized larvae, spherites did not show a preferential location within a particular part of the cell.

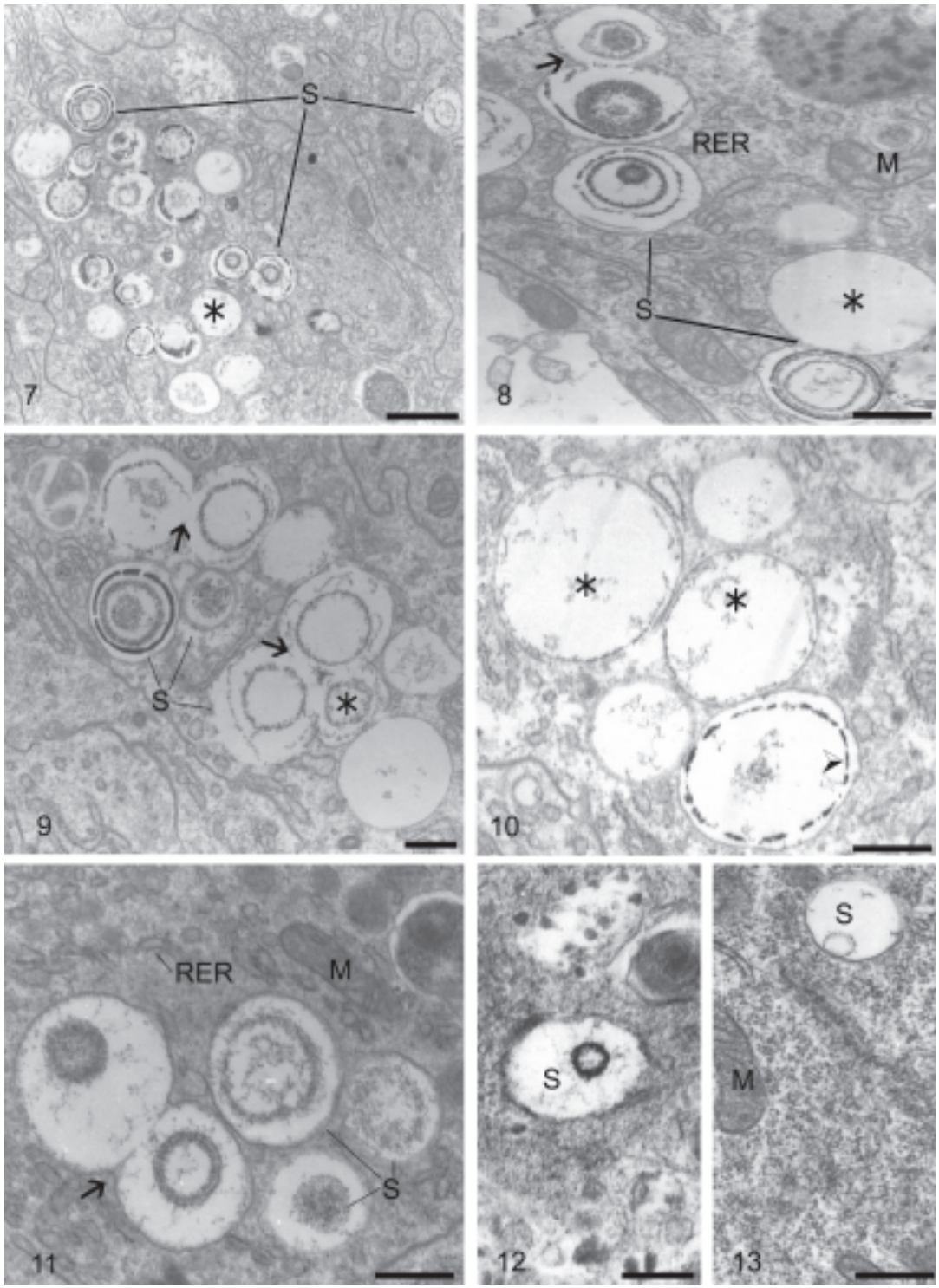
In midgut epithelial cells of parasitized *D. saccharalis* larvae, we observed more spherites in epithelial cells, especially in columnar cells of the anterior midgut region than in other cell types. The abundance of spherites in the anterior midgut region in Diptera, in a normal situation of insect development, was reported by Nopanitaya and Mish (1974). Cruz-Landim (1971), in studying midgut columnar cells of *T. postica*, related that the accumulation of spherites at the end of larval development causes cellular hypertrophy. A morphometric study of epithelial cells in *D. saccharalis* parasitized by *C. flavipes* showed that parasitism induces cellular increase only in columnar cells of the posterior midgut region and that this process is independent of the increase in abundance of spherites (Pinheiro *et al.,* 2006); thus, we believe that the spherite abundance, in our observations, principally in anterior columnar cells, cannot be related to cellular hypertrophy.

The function of spherites in epithelial cells of the midgut or other insect organs is not yet totally understood. One of the functions postulated is related to cellular degeneration. For example, Turbeck (1974) observed that spherites in midgut epithelial cells appear in larvae before ecdysis and disappear during cellular differentiation. The presence of spherites close to the Golgi complex in midgut secretory cells of *Anystis* sp led Waku and Sumimoto (1974) to propose the participation of this structure in the secretion process. Other authors have reported that this structure is associated with cellular excretion of ions and detoxification (Wright and Newel, 1964; Cruz-Landim, 1971, 2000; Ludwig and Alberti, 1988; Serrão and Cruz-Landim, 1996; Lipovsek *et al.*, 2002, 2004). However, according to Sohal *et al.* (1977), the presence of spherites may be associated with food ingestion or insect age; the richer in metals the food, or the older the larvae, the greater the spherite quantity observed in the columnar cell cytoplasm.

As the spherites containing calcium disappear after pupation, some authors associate the structures with mineralization of the cuticle (Ludwig and Alberti, 1988; Lipovsek *et al.*, 2002). We cannot confirm or discard this association, because our observations in *D. saccharalis* are restricted to the beginning of the fifth instar. As the parasitized and non-parasitized larvae had access to the same diet, we discarded the possibility that the greater number of spherite in the parasitized larvae was related to a variation in the diet of the insect.

Some authors have observed the release of spherites in to the intestinal lumen (Wright and Newel, 1964; Gouraton, 1968 apud Serrão and Cruz-Landim, 1996; Cruz-Landim, 1971). The same phenomenon was observed in Malpighian tubules of some insects (Hazelton *et al*., 1988; Spring and Felgenhauer, 1996). It is known that Malpighian tubules are responsible for the maintenance of the hydric and ionic equilibrium of the insect (Chapman, 1998). The spherites are related to the process of rapid transport of fluids and to excretion of the heavy metals, organic materials, and inorganic materials stored by spherites (Hazelton *et al.*, 2001). It is also known that midgut columnar cells aid goblet cells in ionic homeostasis and metabolite absorption by intracellular routes (Lehane and Billingsley, 1996; Terra *et al*., 2006). Recently, Fiandra *et al*. (2006) observed that the transport of electrolytes, including calcium ions, by paracellular routes also occurs in Lepidoptera midgut columnar cells equivalent to that proposed for Malpighian tubules (Beyenbach *et al.*, 2000), exercising intracellular modulation mechanisms of this process in the midgut.

We believe that the parasitism of *D. saccharalis* by *C. flavipes* may be provoking ionic disequilibrium, which induces an increases in the number of spherites in the columnar cells; furthermore, the presence of innumerable spherites in anterior midgut columnar cells point to the importance of these structures in ion transport by an intracellular and/or paracellular route in this



FIGURES 7-13. Columnar cells of the anterior (Figs. 7-11) and the posterior regions (Figs. 12-13) of the midgut of *Diatraea saccharalis* larvae parasitized by *Cotesia flavipes*: spherite (S); rough endoplasmic reticulum (RER); mitochondria (M); fusion of spherite membranes (arrow); flocculated material (✳). **Fig. 7.** Spherites of heterogeneous morphology. Bar = 1μm. **Fig. 8.** Spherites of heterogeneous morphology. Bar = 0.5μm. **Fig. 9.** Innumerable spherites with electron-lucent lamellas among flocculated material, and few spherites with electron-dense lamellas. Bar = 0.5μm. **Fig. 10.** Spherites with varied morphology; concentration of electron-dense material (arrowhead) near vacuolar membrane of spherite. Bar = 0.5μm. **Fig. 11.** Spherites with thick structured lamellas, a few being electron-dense. Bar = 0.5μm. **Fig. 12.** Spherite with electron-dense lamella. Bar = 0.25μm. **Fig. 13.** Spherite with electron-lucent lamella. Bar = 0.5μm.

midgut region, a subject which requires more research. Consistent with our findings, it was recently verified that midgut spherites represent an ionic barrier regulating the quantity of metal ions that enter the organism of invertebrates through the alimentary tract (Pigino *et al*., 2006).

In Malpighian tubule cells of *Acheta domesticus,* direct interaction was observed between spherites and mitochondria (Hazelton *et al.*, 2001); the authors postulate that inorganic phosphate and calcium ions must be passing between the outer membrane of mitochondria and the membrane surrounding the spherites and, therefore, rendering this intimate association favorable for both organelles. In *D. saccharalis* we did not observe an association between spherites and mitochondria, which suggests that the ionic movement must be occurring by other mechanisms.

Although the fusion between spherite membranes has been frequently observed in midgut columnar cells of parasitized insects, the reason for this fusion is still not known. In columnar cells of bees, the fusion between spherites and autophagic vacuoles, eliminated together to the intestinal lumen, has been observed (Serrão and Cruz-Landim, 1996; Lipovsek *et al.,* 2002).

In parasitized larvae, the numerous spherites observed showed wide variability in the morphology of its content. The majority of the spherites had low densities, contained sparse flocculated material concentrated on the periphery of the vacuolar membrane, and formed a discrete electron-dense layer or a few concentric lamellas. However, in non-parasitized larvae, the few spherites observed contained thick and contrasting lamellas. We believe that the less structured spherites may be younger spherites that were formed as a result of metabolic alterations, especially in the ionic equilibrium, of the host insect during the six-day period of parasitism. The morphological variability of these spherites, especially in parasitized larvae, may also be attributed to instability in the content of these structures as a consequence of ionic alterations related to metabolism.

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