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Extracellular Symbiosis of a Yeast-Like Microorganism Within *Comperia merceti* (Hymenoptera: Encyrtidae)

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Abstract

Adult female *Comperia merceti* (Compere) inject yeast-like microorganisms into oothecae of the brownbanded cockroach, *Supella longipalpa* (F.), during oviposition. A small, non-budding form of the yeast occupies the reservoir of the adult female parasitoid's poison gland and accompanies injection of the egg and venom into the host ootheca. Filamentous forms of yeast cells are found in the gut of first-instar parasitoid larvae. When parasitoid larvae reach the third instar they have large quantities of extracellular yeast in the hemolymph and in the gut. Adult male and female parasitoids retain all yeast in the hemolymph and gut, except those yeast cells concentrated in the adult female poison gland reservoir. The chronic, systemic, and non-pathogenic character of this organism implies a symbiotic relationship with *C. merceti*. Ultrastructure and distribution of the yeast cells are discussed as they relate to the biology of *C. merceti*.

Keywords: *Comperia merceti*, *Supella longipalpa*, yeast, ultrastructure, symbiosis

1. Introduction

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Comperia merceti (Compere) is a gregarious, host-specific, oothecal parasitoid of the brownbanded cockroach, *Supella longipalpa* (F.) (Dictyoptera: Blattellidae). Female *C. merceti* deposit an average of 14 eggs into an *S. longipalpa* ootheca, and developing parasitoid larvae usually consume the entire oothecal contents (Lawson, 1954). Basic biology of *C. merceti*, including longevity, fecundity, mating and oviposition behavior, sex ratio, and host specificity, was reported by Lawson (1954) and Gordh (1973). During biological studies at the University of California Riverside, an extracellular yeast-like microorganism was discovered within body tissues and reproductive organs of healthy *C. merceti*.

Yeast-like symbionts are well documented in several families of Homoptera and Coleoptera (Brooks, 1963; Buchner, 1965). These symbionts are located within specialized midgut or fat body cells, extracellularly in the hemocoel, in the gut lumen, or housed in mycetomes attached to the alimentary or reproductive systems (Buchner, 1965; Henry, 1967; Cooke, 1977). Utilization of yeast as a food source is well known for many insects, including honeybees and ants (Cooke, 1977; Batra, 1979). Among parasitic hymenoptera, mechanical transmission of yeasts between hosts via a contaminated ovipositor has been established (Steinhaus, 1963; Larsson, 1979). However very few cases of yeast-parasitoid symbiosis have been reported. Middeldorf and Ruthman (1984) documented the transovarial transmission of yeast-like endosymbionts to progeny of *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae). *Adelura (=Alysia) apii* (Curtis), a braconid parasitoid of the larval celery fly, *Acidia heraclei* L., has a permanent, though poorly known, relationship with a yeast-like fungus requiring both the parasitoid and host to complete its life cycle (Keilin and Tate, 1943).

Currently much research is focused on effects of female parasitoid secretions in the host-parasitoid relationship. Secretions injected during oviposition include venom and accessory gland products which may contain virus or bacteria-like particles. Many of these have been implicated in specific cases of host regulation (Vinson and Iwantsch, 1980; Stoltz, 1986). Injection of these materials usually benefits the parasitoid egg and early instars by paralyzing the host, suppressing encapsulatin and/or any immune response (Rizki and Rizki, 1984; Guzo and Stoltz, 1987). In addition, a maternally transmitted bacterial infection was found linked to the "son-killer" trait in *Nasonia vitripennis* (Walk.) (Hymenoptera: Pteromalidae) in which approximately 80% of all male progeny dies (Huger et al., 1985).

The objective of this study was to determine various aspects of the relationship of the yeast-like symbiont to *C. merceti*, including its life cycle, ultrastructure, and mode of transmission.

2. Materials and Methods

S. longipalpa colonies, maintained on Purina Dog Chow® and water, provided host oothecae for laboratory cultures of *C. merceti*. Mated female parasitoids were provided oothecae and resulting parasitized oothecae were held for progeny emergency. Parasitized oothecae were identified by the pedicels of deposited eggs protruding externally through the oothecal wall. Cockroach and parasitoid cultures were maintained in the dark at ca. 28±1°C.

Fresh preparations

All developmental stages of *C. merceti* were dissected alive in Ringers saline and examined for yeast cells. Number of dissections ranged from 50–100 each for larvae, prepupae, pupae and adult males of *C. merceti*. Approximately 500 dissections of adult females were performed. Parasitized and unparasitized *S. longipalpa* embryos were examined in a similar manner (50 individuals each). When possible, individual organs were isolated, washed, and separately dissected to further locate the yeast. These included portions of male and female digestive system, reproductive system, fat body and larval salivary glands. Freshly deposited eggs were washed in saline, ruptured under a cover slip, and inspected for yeast cell location and morphology.

Histology

Parasitized *S. longipalpa* embryos, *C. merceti* larvae, pupae and adult abdomens were processed in Paraplast®. Tissue was dissected live or placed in Formol-alcohol (Humason, 1967) for 24 hr, washed and dehydrated in ethanol, cleared in toluene, and embedded in Paraplast. Serial sections of 8 μm thickness were stained with Delafield's hematoxylin, Weigerts Iron Alum hematoxylin, and counter-stained with eosin (Humason, 1967). A DePalma and Young (1963) modification of the Hotchkiss-McManus method (McManus, 1948) was used to reveal complex carbohydrates in yeast, rendering the cells more visible in sections.

Scanning Electron Microscopy (SEM)

To facilitate SEM of deposited *C. merceti* eggs, parasitized oothecae were opened and the embryonic contents flushed out with Ringers saline. Deposited eggs remained anchored to the inner oothecal wall by the pedicels

which protrude through the wall and are externally visible on the ootheca. Portions of oothecal wall with attached eggs were fixed in 70% ethanol, dehydrated in a graded ethanol series, and critical-point dried. Specimens were mounted on aluminum stubs, coated with gold-palladium, viewed on a JEOL-JSM scanning electron microscope (15 KV), and photographed with Polaroid® 55 P/N film.

Transmission Electron Microscopy (TEM)

Third-instar *C. merceti* larvae (largest instar by weight) were dissected directly into a fixative of 3.0% glutaraldehyde in 0.1 M cacodylate buffer. Large pieces of tissue were removed leaving a suspension of hemolymph, fat body and additional cellular debris. The suspension was centrifuged to a pellet, supernatant discarded, and the pellet resuspended in fresh fixative for 3 hr. A similar procedure was followed for postfixation in 1.0% Osmium tetroxide, washing in buffer, and dehydration in a graded acetone series. Fixed cells were processed to Spurr's Low Viscosity resin in a pellet, thin-sectioned on an ultramicrotome, and poststained with uranyl acetate and Reynolds lead citrate stain (Reynolds, 1963). Sections were viewed and photographed on a Hitachi H-500 transmission electron microscope.

Abdomens of adult female *C. merceti* were processed to Spurr's Low Viscosity resin following the same fixation procedures as above. Thick sections were mounted and stained with methylene blue for light microscopy.

3. Results and Discussion

Cell morphology and distribution

Morphology of yeast cells found in *C. merceti* varied with respect to host developmental stage and location within specific organ systems. The most common vegetative yeast cell type was found in the hemolymph of late third-instar larvae and remained in the hemolymph through the adult stage. Examination of clear cuticular regions of living second- and third-instar larvae (Fig. 1) with a compound microscope, revealed yeast cells circulating in the hemolymph. These typically elongate cells averaged 9±1 μm in length, 2±.05 μm in width (Table 1). They often exhibited bipolar budding (Fig. 2). Frequent bipolar budding of individual cells often produced apiculate (lemon-shaped) forms (Phaff et al., 1978). No cross-wall formation was apparent which indicated bud-fission was not taking place (Barnett et al., 1983). Sexual structures, such as ascospores, teliospores, or basidia were not observed, suggesting the organism was in the Deuteromycotina. Cells

Table 1. Yeast cell morphology and anatomical location in developmental stages of *Comperia merceti* and parasitized *Supella longipalpa*

Developmental stage	Gross cell morphology	[a]Average cell size (x±SD) (μm)	Anatomical location
Egg	elongate, no apparent budding	8.5±1	external chorion
1st-Instar larva	elongate, hyaline filaments	32±1 (7.8–80.6)	Developing gut
2nd-Instar	elongate, some budding filaments, some branching	35±1 (7.8–92)	gut hemolymph in late 2nd instar
3rd-Instar	budding cells common, some filaments in gut only	18±1 (7.8–36.4)	gut and hemolymph
3rd-Instar larva (after) voiding meconia)	elongate, budding common	9±1 (7.5–10.4)	gut and hemolymph
Prepupa	elongate, single and budding cells	9±1	gut and hemolymph
Pupa	same as prepupa	9±1	gut and hemolymph
Adult male	elongate, single budding cells	9±1	gut, fat body, hemolymph
Adult female	elongate, single budding	9±1	gut, fat body, hemolymph
	elongate, no budding	6±1	poison gland reservoir, common oviduct, 2nd accessory glands?
	Pseudomycelium		Reservoir of poison gland, exclusively
Parasitized *Supella* embryo after 48 hr	elongate, hyaline	15±1	Within degenerating body tissue of embryo

[a] Range is included for developmental stages in which long yeast cells (found in gut) affect average cell size.

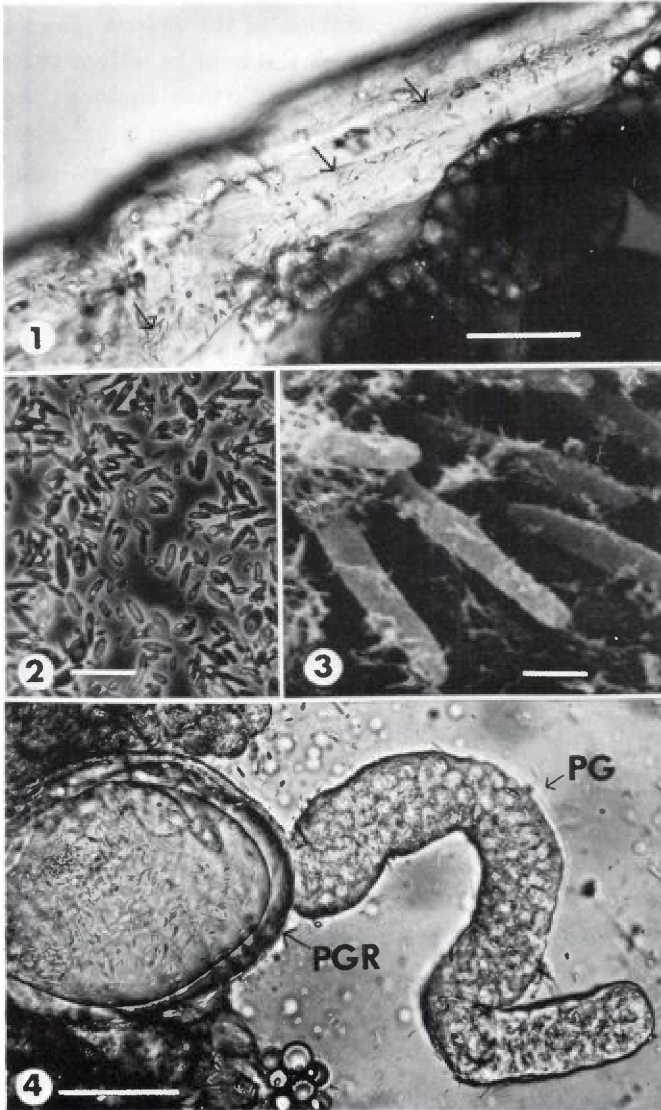
appeared hyaline and vacuoles were visible in the cytoplasm under phase-contrast microscopy.

Light microscopic examinations (including squashes) of *C. merceti* oviposited eggs (up to 96 hr old) showed no yeast within the egg. However, SEM of eggs attached to the inner oothecal wall revealed cells coating the chorion (Fig. 3). Depending on embryonic development of the cockroach at parasitization (amount of yolk vs. embryonic tissue), yeast were difficult to observe in fresh dissections until 24 hr post-parasitization (*C. merceti* eggs hatch from 48–96 hr (Lawson, 1954)). Squashes of first-instar larvae revealed yeast cells averaging 32±1 μm in length with some of the longest "filaments" measuring up to 80.6 μm (Table 1). Production of filaments by yeast is a type of primitive pseudomycelial growth where filaments resemble true mycelia (Phaff et al., 1978). However, unlike true mycelia, cells elongated through budding (vs. cross-wall formation) and buds remained attached to the mother cell instead of breaking away.

Yeast cell morphology in second-instar *C. merceti* (days 6–8) was variable (Table 1) with some filamentous forms exhibiting simple branching. Notable quantities of budding cells were evident in late second-instar and early third-instar larval hemolymph. In addition, average cell size in third-instar larvae began to decrease (Table 1) with filamentous forms appearing exclusively in the gut. After third-instar larvae voided meconia, yeast cell length, including those found in the gut, did not exceed 10.4 μm (Table 1). Cells were predominantly elongate to apiculate. Ca. 50% of the population appeared to be budding (visual estimation). Throughout the pupal stage, yeast were found in both gut and hemocoel, where mean cell length remained about 9±1 μm with budding and single cells common. No mycetomës or mycetocytes were found in the *C. merceti* alimentary system in any developmental stage.

Distribution and morphology of yeast in adult male *C. merceti* was similar to that found in the pupal stage. Elongate single and budding cells were observed in the gut as well as hemocoel where they appeared to concentrate in the abdomen. No yeast were located within other mature organs including male reproductive structures.

Dissections of adult female *C. merceti* revealed yeast cells in the midgut and hemolymph. As in males, yeast in the hemolymph were typically found in the abdomen, often among the fat body. These cells were morphologically identical to budding and single cells in pupal hemolymph. During examinations of the female reproductive tract (fresh preparations; light microscope), the poison gland reservoir (Figs. 4, 6) frequently ruptured and released a



Figures 1–4. (1) Whole wet mount of late 2nd-instar *C. merceti* larva. Yeast cells in hemolymph (arrows). Bar = 40 μm.

(2) Wet mount preparation of centrifuged hemolymph from *C. merceti* larva. Single, budding, apiculate-shaped cells. Bar = 20 μm.

(3) SEM of yeast cells coating surface of deposited *C. merceti* egg. Bar = 2.5 μm.

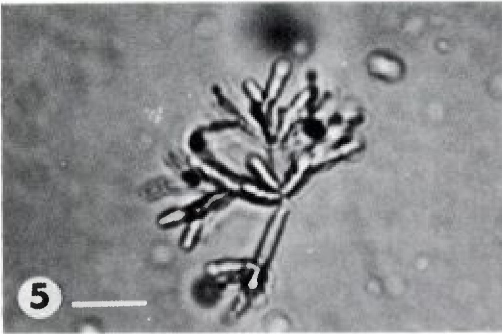
(4) Wet mount of poison gland (PG) and reservoir (PGR). Yeast cells present within inner membrane. Bar = 90 μm.

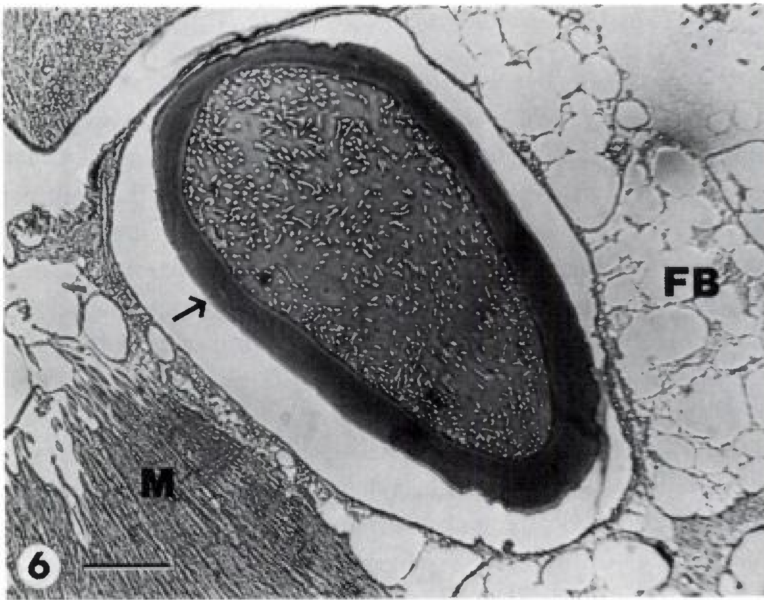
dense mass of yeast cells. Cells were elongate, non-budding, and averaged 6±1 μm in length. In a fresh preparation of the poison gland and reservoir (Fig. 4), yeast cells were visible through the hyaline wall of the reservoir. An additional internal membrane appeared to further enclose the yeast. When the wall was ruptured, yeast cells poured out with a colorless, viscous secretion, presumably venom produced by the large poison gland. Most cell counts fell within a range of 300–700 cells per reservoir. However, reservoirs with greater than 1000 and as few as 50 cells were observed. No trends in relative concentrations of yeast per poison reservoir were found between newly emerged females (virgin or mated), females allowed to oviposit for various lengths or time, females offered or deprived of honey, or senescent females. Within the poison reservoir, an intricate pseudomycelium frequently developed in addition to singular yeast cells. Unlike the long, simple filaments found in the early larval midgut, pseudomycelia (one, rarely two, per reservoir) were strongly branched and readily flowed from the ruptured reservoir with other yeast cells (Fig. 5).

Histological sections of the female reproductive system also showed yeast within the poison reservoir (Fig. 6). They may have been in the common oviduct and secondary accessory glands, however, yeast were not expelled from secondary accessory glands during dissections because the thick gland reservoir walls did not easily rupture.

Ultrastructure

Yeast can be identified by distinctive cell walls, often associated with other structural layers to form a characteristic cell envelope (Arnold, 1981). The microorganisms in *C. merceti* had well-differentiated envelopes and contained cytoplasmic organelles typical of yeast. The most external component of the cell envelope consisted of a capsular material, or slime layer, irregular in width, and well developed in most cells examined (Figs. 7, 8). Garrison (1981) described stabilizing microfilaments in slime layers of some yeast species, but these were not apparent in *C. merceti* yeast. Paraffin sections of second-instar larvae, subjected to a modified PAS technique (Hotchkiss-McManus method for fungi; Humason, 1967), stained yeast pink, indicating the polysaccaride composition of the slime layer (Bacon, 1981). Arnold (1981) hypothesized that yeast cells with capsules of slime layers were more "bulky" and not easily phagocytized by animal cells. Perhaps this characteristic allows some yeast cells to escape nodulation or destruction by *S. longipalpa* hemocytes present in later stage embryos. Graham (1967) suggested





Figures 5–6. (5) Pseudomycelium from poison gland reservoir of *C. merceti* (wet mount). Bar = 10 μm.
(6) Thick plastic section of poison gland reservoir (arrow) with yeast in lumen. Fat body (FB), muscle (M). Bar = 25 μm.

the mucus coat of spore-like yeast in transmission organs of some beetles prevents their digestion by enzymes in the larval gut. Similar mechanisms may protect yeast cells in the *C. merceti* larval gut. Cells devoid of slime layers had an electron dense, microfibrillar zone in the outer-most area of the true cell wall (Fig. 9). While this could be due to cell age, thickness

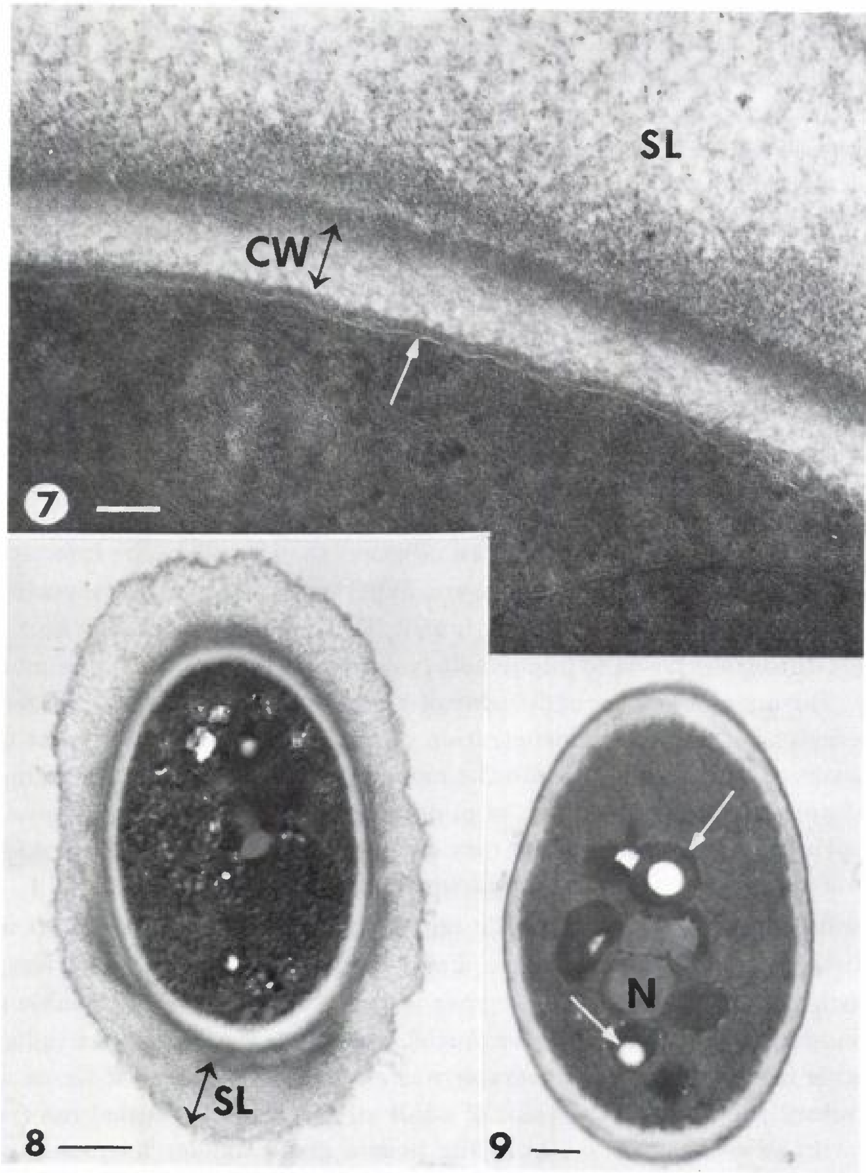
and opacity of the cell wall also varies with different fixation and staining techniques (Garrison, 1981).

Between the cell wall and plasma membrane was a periplasmic space. In *C. merceti* yeast, this space was discernible as an irregular, electron dense area, adjacent to the plasma membrane (Fig. 7). Although tripartite in structure (Garrison, 1981), the plasma membrane appeared as a sinuating bilayer. Vacuoles, visible with phase contrast as bright, white spots, varied in size and number (up to 5 per cell) (Fig. 9), and were bound by a single membrane, the tonoplast (Arnold, 1981). Nuclei were spherical and indistinct, and mitochondria were present, though not abundant.

Yeast life cycle

Yeast, concentrated in the *C. merceti* poison gland, were injected along with stored secretory products (venom), into the *S. longipalpa* egg during oviposition. In addition to transmission as a contaminant of the venom injected intentionally, pressure of an egg passing through the common oviduct (past the efferent duct of the poison gland reservoir) may force yeast out of the reservoir onto the egg chorion. Internal, membranous, symbiont-filled pouches and cuticular modifications function as egg-smearing organs insuring symbiont transmission in various species of Hemiptera, Coleoptera, Diptera and Hymenoptera (Buchner, 1965; Francke-Grosmann, 1967; Henry, 1967; Cooke, 1977). Most of these symbionts are bacteria or yeast-like, and in many cases, studies have established their role in providing nutrients essential to normal host growth (Koch, 1967). Insects that depend upon inoculating mutualistic fungi into potential food sources (for assisting digestion of substances such as cellulose), have been shown to carry the infective stage in structures near the ovipositor (Buchner, 1965).

First-instar *C. merceti* larvae began feeding immediately (Lawson, 1954). Although inoculated yeast of *C. merceti* did not heavily populate the *S. longipalpa* egg, cells were found in embryonic tissues and on the parasitoid egg surface. Ingestion of a few yeast cells may be sufficient to inoculate the larval parasitoid gut. The gut environment of *C. merceti* supported a thriving yeast population. The pleomorphic and filamentous yeast forms, discovered in the developing gut of early instars, have been noted in other insect-symbiont relationships, and may be correlated to nutritionally rich gut contents (Keilin and Tate, 1943; Buchner, 1965; Henry, 1967). The larva of *Adelura (=Alysia) apii* (Braconidae) feeds on the fungal-filled hemolymph of its host, the celery fly, *Acidia heraclei* (Keilin and Tate, 1943). This braconid is suspected of



Figures 7–9. (7) TEM of yeast cell showing layers of yeast cell envelope. The periplasmic space is the thin, electron dense area between the plasma membrane (single arrow) and the cell wall (CW). Slime layer (SL). Bar = .1 μm.
(8) Yeast cell; Slime layer (SL). Bar = .5 μm.
(9) Yest cell with vacuoles (arrows) and no slime layer; Nucleus (N). Bar = .5 μm.

transmitting the yeast-like organism to the host larva during parasitization. The midgut of *A. apii* is filled with a mycelium of septate hyphae, presumably a different stage in the microorganisms' life cycle. Several *Donacia* species (Coleoptera: Chrysomelidae) provide an external mass of bacterial symbionts with each deposited egg (Buchner, 1965). Larvae hatch, consume the bacteria (along with the eggshell) and within hours, produce filamentous forms of the symbiont (up to 15 μm) in the host gut.

In general, symbiotic yeast-like organisms transmitted to insect progeny within the ovarian egg, infect mycetocytes among such diverse tissues as midgut epithelium, fat body, and malpighian tubules (Brooks, 1963; Buchner, 1965; Henry, 1967). As sexual maturity approaches, symbionts in females migrate to and invade developing ova. The stimulus causing migration to the reproductive system is not clear, however, Eberle and McLean (1982) provide evidence that hormonal gradients within the hemolymph attract the bacteria-like symbiont in *Pediculus humanus* L. (Anoplura) to the ovaries. Egg-smearing devices located adjacent to ovipositors are infected by symbiotic yeast in a different manner. Whether housed in gut mycetomes or mycetocytes, or free in the gut lumen, these symbionts often reach the hindgut during the larval to pupal molt (voiding of waste products) (Buchner, 1965). During the late second-instar of *C. merceti*, yeast cells appeared in the hemolymph. No direct penetration of midgut epithelium by yeast cells was observed. Mode of entry into the hemocoel is unknown. However, ingestion of yeast during early stages of midgut development may locate yeast in other primordial tissues, or yeast may enter the hemocoel during the midgut apolysis and histogenesis associated with molting.

Glandular portions of the female reproductive system of *C. merceti* were not distinct in pupal dissections. Forty-eight hours before adult females emerged, ovarioles with developing ova were visible with an identifiable poison gland and reservoir. However, nuclei and collecting ducts were indistinguishable in the gland. The reservoir was an amorphous mass of tissue void of secretory products. Dissections of adult virgin females revealed reservoirs filled with yeast. Secretions from the poison gland and/or hormones activated at sexual maturity may be responsible for rapid yeast growth (Koch, 1955). *Coptosoma scutellatum* Geoffroy (Hemiptera) experiences an increase in growth rate of its pleomorphic bacterial symbionts at the onset of sexual maturity and Henry (1967) suggests its abundance within a terminal hindgut bladder serves as infective material during oviposition.

Yeast cells in the poison gland reservoir were morphologically homogenous, except for one (rarely two) pseudomycelium. Individual hyaline cells were relatively short (about 6 μm in length) and were never observed budding. This is characteristic of transmission forms present in egg-smearing organs of other insect species. Buchner (1965) described yeast in transmission organs of cerambycids as smaller, rounder and "spore-like" due to less favorable growing conditions.

The chronic and systemic infection of this yeast in healthy *C. merceti*, and the accumulation of a possible "transmission form" in the poison gland reservoir insuring yeast transfer to progeny, suggests a symbiotic relationship. The physiological role the yeast plays in *C. merceti* can only be determined through symbiont elimination. Experimentally, yeast symbionts provide nutritional benefits (B vitamins) to hosts (Koch, 1967). However, because the *C. merceti* yeast is injected into the cockroach embryo along with the parasitoid egg, its possible function in parasitoid egg protection cannot be ignored. Reproductive tract materials transferred during oviposition in some parasitoids clearly interfere with hemocytes responsible for encapsulation of parasitoid eggs (Rizki and Rizki, 1984; Stoltz, 1986). Very late stage *S. longipalpa* embryos (30–35 days) were successfully parasitized by *C. merceti* (Lawson, 1954). Within 6 hr following parasitization, overall rapid tissue histolysis occured in the embryo (LeBeck, unpublished data). This is not attributed to parasitoid feeding because *C. merceti* hatches from 72–96 hr after oviposition (Lawson, 1954). This implicates injected venom produced by the poison gland, the yeast, or other secretions from the reproductive tract as the cause of rapid death. Analysis of individual effects of these components on the cockroach embryo may determine which is responsible for the histolysis. Recently, Verrett et al. (1987) reported hemocyte destruction, presumably from the toxin of a pathogenic yeast, in *Periplaneta americana* (L.) (Dictyoptera: Blattidae). Future study of this parasitoid-host relationship should include affects of the yeast, its by-products, or other reproductive gland secretions, such as venom, on hemocytes in pre-hatching cockroaches. In addition, eliminating the yeast from *C. merceti* may also indicate its role in larval parasitoid nutrition.

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