

An Investigation of Harpellales (Trichomycetes) in New York State Blackflies (Diptera: Simuliidae)

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This research project investigates the distribution and prevalence of trichomycete fungi present in blackfly larvae and adults in the region of Cambridge, New York. In blackfly larvae, two genera of Harpellaceae, *Stachylina* and *Harpella*, were observed in midguts, and four genera of Legeriomycetaceae—*Pennella*, *Genistellopora*, *Simuliumyces*, and *Smittium*—in hindguts, with a total of seven species recorded. This is the first published report of *Stachylina* spp. in blackflies. Field data indicated that sampling date, host species, and location were all important variables affecting trichomycete prevalence in larvae. We report the successful laboratory infection of blackfly larvae with *in vitro* cultured *Smittium simulii*, and production of *H. melusinae* and *G. homothallica* trichospores from cysts dissected from the ovaries of infected flies. © 1996 Academic

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KEY WORDS: Trichomycetes; Harpellales; fungi; Simuliidae; ovarian cysts.

INTRODUCTION

Fungi in the class Trichomycetes are cosmopolitan, obligate inhabitants of the guts of arthropods, including aquatic insects such as flies (Diptera), mayflies (Ephemeroptera), and stoneflies (Plecoptera) (Lichtwardt, 1986). Blackfly (Simuliidae) larvae have been reported as hosts of trichomycetes in the order Harpellales in France (Léger and Duboscq, 1929), England (Moss, 1970), Canada (Frost and Manier, 1971), the United States (Lichtwardt, 1972), New Zealand (Crosby, 1974; Williams and Lichtwardt, 1990), Japan (Lichtwardt *et al.*, 1987), and Australia (Lichtwardt and Williams, 1990, 1992). Two species in the family Harpellaceae have been recorded from blackfly midguts and 17 species in the family Legeriomycetaceae from hindguts (Lichtwardt, 1986; Williams and Lichtwardt, 1990; Lichtwardt and Williams, 1990). Moss and Descals (1986) observed that fungal cysts associated with blackfly ovaries and egg masses, previously referred to as

“phycomycetes” (Garms, 1975; Undeen and Nolan, 1977; Yeboah *et al.*, 1984), were actually trichomycetes. This discovery by Moss and Descals (1986) revealed for the first time that trichomycetes could have a parasitic phase in their life cycle. Infection of the ovaries is so extensive that mature eggs are rarely found (Yeboah *et al.*, 1984). In addition to their commensal existence in the gut, maintained by passage of trichospores from larva to larva, trichomycetes could also invade and destroy ovarian tissues, theoretically infecting blackfly larvae with trichospores produced therefrom (Fig. 1).

This research project was undertaken to investigate the trichomycetes present in blackfly larvae and adults in eastern New York state (USA). We report distribution/prevalence records for trichomycete species (including the first record worldwide of *Stachylina* in blackflies), the successful laboratory infection of blackfly larvae with cultured trichomycetes, and production of trichospores from cysts dissected from infected ovaries.

MATERIALS AND METHODS

In order to survey which species of Harpellales were regionally present in blackfly larvae, late instars were collected in the spring and summer of 1995 from four locations near the village of Cambridge, New York: Camden Creek, Fish Hatchery Stream, Whittaker Brook, and Carter Pond Outlet (the origin of Whittaker Brook). Prevalence of trichomycetes was recorded following dissection and examination (450×) of midguts (i.e., peritrophic membrane) and hindguts following the procedures of Lichtwardt (1986).

Laboratory trials were conducted to infest blackfly larvae with cultures of the hindgut trichomycete, *Smittium simulii*. This species, obtained from local *Simulium tuberosum* larvae (Table 1, column 5), was cultured at 14°C on brain–heart infusion agar following Lichtwardt (1986). Mid-to-late instar *Simulium vittatum* larvae from Fish Hatchery Stream, a population known to be uninfested with any hindgut trichomycetes (Table 1, column 7), were placed in a beaker of aerated stream water at 14°C, fed *Chlorella*, and treated with trichospores and sporulating thalli from several petri dishes. Three larvae were dissected each day for the presence of *S. simulii* thalli.

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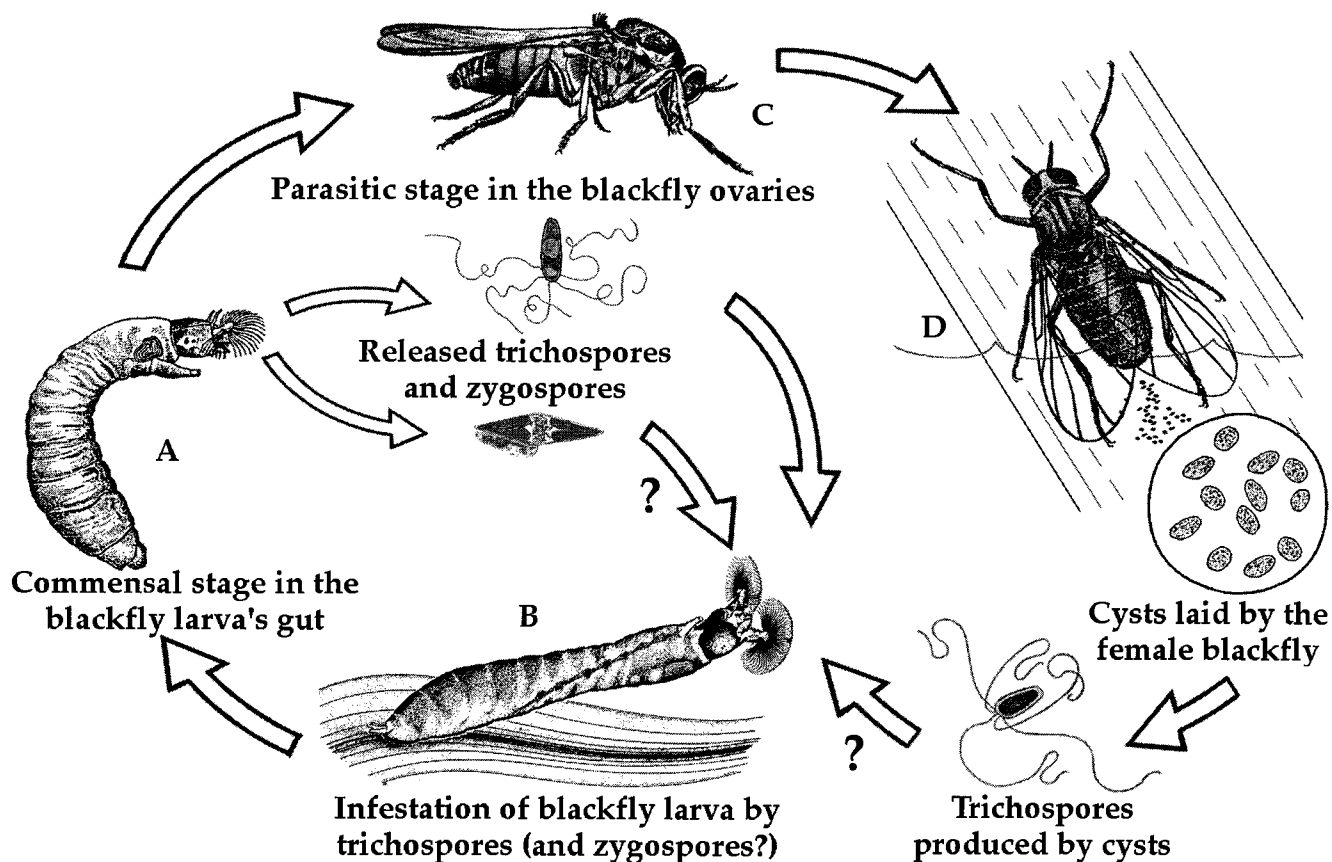


FIG. 1. Life cycle of Harpellales (Trichomycetes) in Simuliidae (here *Genistellospora homothallica*). Transmission from larva (A) to larva (B) occurs either by asexually produced trichospores or, hypothetically, by sexually produced zygospores. Females emerging as infected adults (C) deposit cysts in mock oviposition (D). Trichospores developing from these cysts theoretically infest larvae. (Figs. 1A and 1C redrawn from Rubtsov (1989); Fig. 1B redrawn from Currie (1986).)

Following a similar protocol, a second beaker was also treated, but at a higher dosage of trichospores (ca. $10\times$).

To determine the prevalence of trichomycete cysts in adult female *Simulium* spp., 1692 and 466 flies were collected in the field during April–July in 1987 and 1993, respectively, at oviposition sites in the local Cambridge, New York area; in 1995, 30 *S. vittatum* and 207 *Cnephia dacotensis* females were similarly examined. Trichospores were produced from cysts collected from infected flies in 1993 following either of two techniques. In the first method, cysts were placed in 60-mm dishes containing simple water agar (2% agar w/w in 100 ml distilled water); to inhibit bacterial growth, 0.1 ml of a stock antibiotic solution (40,000 units of penicillin G and 80,000 units of streptomycin sulfate per milliliter of distilled water, filter sterilized) was added per liter of medium. The second method, which was ideal for viewing and photographing cysts and stages of germination, involved placing the cysts directly on a microscope slide in a drop of distilled water containing 0.1 ml of the above-mentioned stock antibiotics per liter; these slides, supported by glass rods, were held in a petri dish with a small amount of water kept in the bottom of the dish.

RESULTS AND DISCUSSION

Two genera of Harpellaceae, *Stachylina* and *Harpella*, were observed in midguts, and four genera of Legeriomycetaceae—*Pennella*, *Genistellospora*, *Simuliomyces*, and *Smittium*—in hindguts, with a total of seven species recorded (Table 1). The most significant find was a *Stachylina* sp. which closely resembles *Stachylina longa*, but its thallus contains more generative cells (>8) and its mature trichospores were slightly longer (27–32 μm) and wider (7 μm) (Fig. 2 and 3). While *Stachylina* spp. are well known from other aquatic dipterans (Chironomidae and more rarely in Psychodidae and Thaumaleidae) (Lichtwardt, 1986; Lichtwardt and Williams, 1990), this represents the first report of this genus in blackflies. *Stachylina* was noted in two *S. vittatum* larvae from Carter Pond Outlet, one of which was involved in prevalence calculations (column 4, Table 1). In previous sampling on May 20, 1993, we had also observed a *S. vittatum* larva at Thurber Pond Outlet (Cambridge, NY) with a *Stachylina* sp. (Fig. 4) which was similar to *Stachylina nana*. The two remaining mature trichospores measured $30\times$

TABLE 1
Trichomycete Prevalence in Species of Blackfly Larvae in the Area of Cambridge, New York

	1	2	3	4	5	6	7
Host species	<i>Simulium vittatum</i>	<i>Cnephia dacotensis</i>	<i>Cnephia dacotensis</i>	<i>Simulium vittatum</i>	<i>Simulium tuberosum</i>	<i>Simulium</i> spp. (not <i>vittatum</i>)	<i>Simulium vittatum</i>
Sampling date (1995)	April 14	April 14	April 24	May 23–31	July 20–August 3	August 8	August 9
Locality	CPO ^a	CPO	WB ^b	CPO	CC ^c	FHS ^d	FHS
Sample size	11	17	24	20	34	17	15
Midgut trichomycetes species							
<i>Harpella melusinae</i>	9%	29%	83%	100%	68%	100%	100%
<i>Stachylina</i> sp.	0%	0%	0%	5%	0%	0%	0%
Hindgut trichomycete species							
<i>Smittium culicis</i> (?)	9%	47%	29%	5%	0%	0%	0%
<i>Smittium simulii</i>	0%	0%	0%	0%	45%	0%	0%
<i>Simulioomyces microsporus</i>	0%	0%	13%	0%	18%	0%	0%
<i>Pennella</i> sp.	0%	0%	4%	10%	0%	0%	0%
<i>Genistellospora homothallica</i>	0%	0%	75%	35%	9%	35%	0%
Total hindgut thalli prevalence ^e	9%	47%	92%	95%	82%	41%	0%

^a Carter Pond Outlet.

^b Whittaker Brook.

^c Camden Creek.

^d Fish Hatchery Stream.

^e Percentage indicated does not represent the sum of the individual prevalences stated for each hindgut species since some hindgut thalli were unidentifiable and multiple infections occurred frequently.

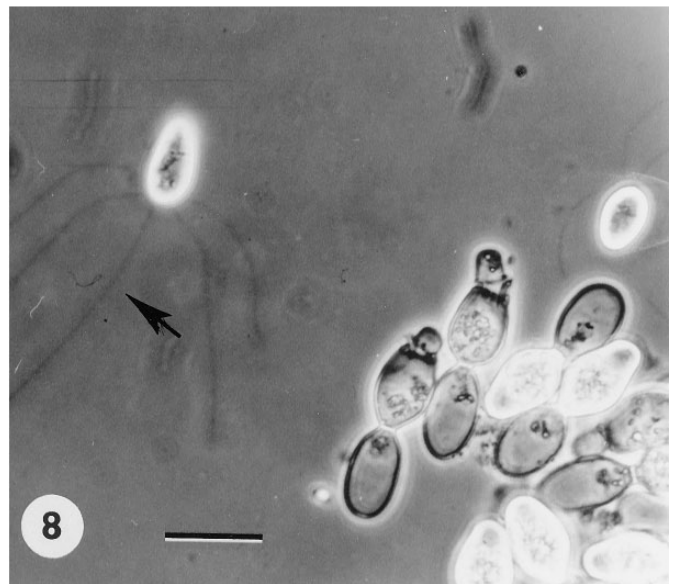
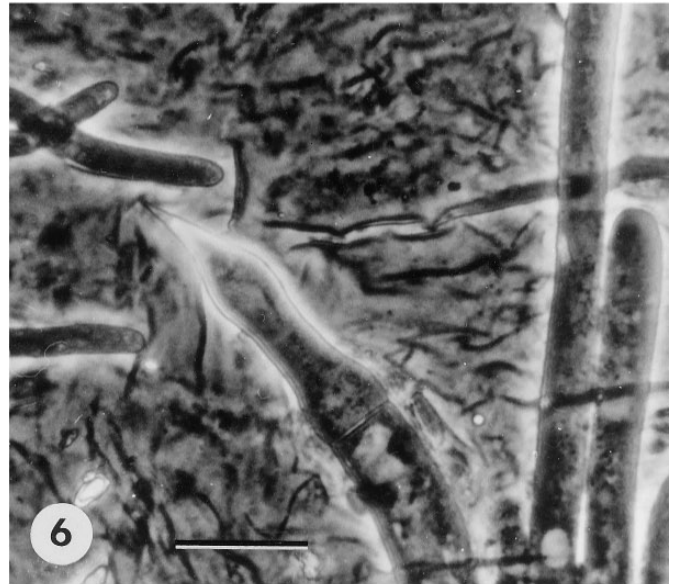
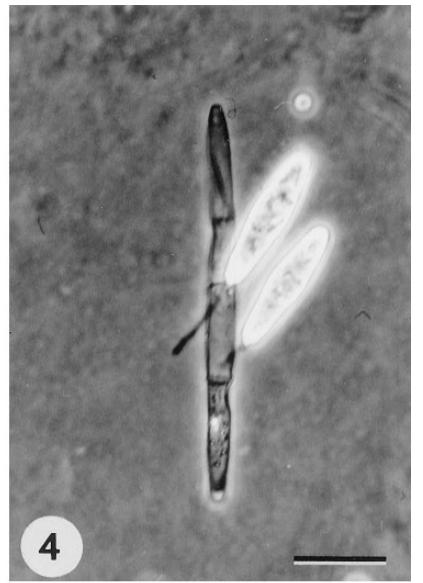
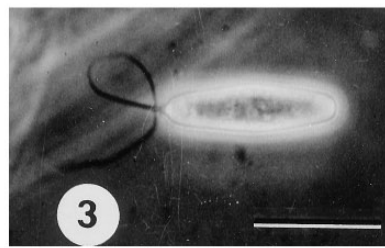
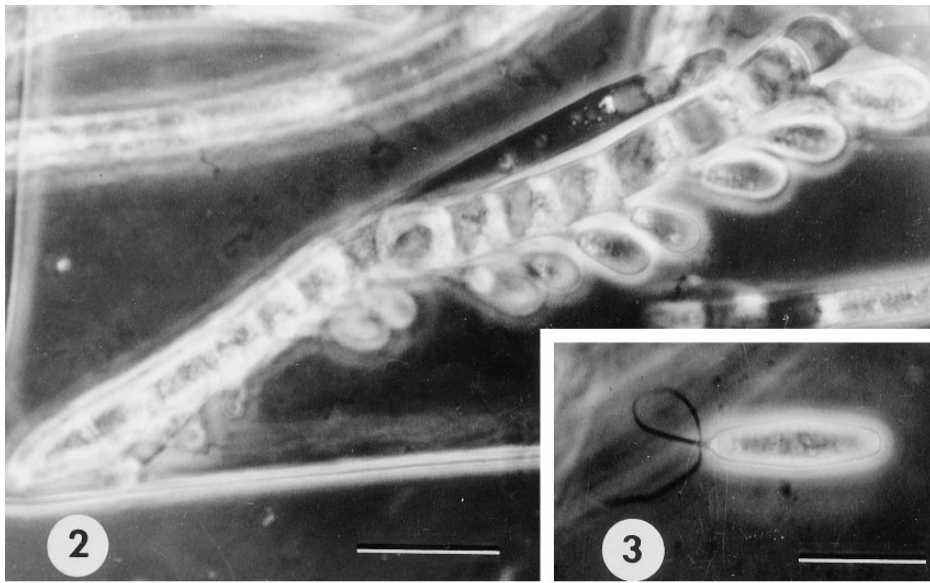
8 µm, produced on a thallus that was 80 µm long by about 6 µm diameter with four generative cells.

All other trichomycete genera and species (*Harpella melusinae*, *Simulioomyces microsporus*, *Pennella* sp., *S. simulii*, *Genistellospora homothallica*, and *Smittium culicis* (?)) represent new records for the eastern United States. *H. melusinae*, a cosmopolitan trichomycete specific to simuliids, was the only species found in all samples. *S. culicis* (Fig. 5) has been frequently reported from Culicidae, but rarely in Simuliidae and Chironomidae (Lichtwardt, 1986). Although the measurements of the specimens found during this survey are within the range described for *S. culicis* (Manier, 1969), the trichospores have a greater length-to-width ratio and appear more elongate (LxW = 22.7–26.0 × 4–5 µm, collar = 8 µm). Since it was not possible to observe the holdfast structures of these specimens, they cannot be definitely classified as *S. culicis*, and thus we refer to our record as *S. culicis* (?).

Very few papers have reported trichomycete prevalence data in blackfly larvae. Our field data indicated that sampling date, host species, and location were all important variables affecting prevalence. Trichomycete prevalence in *S. vittatum* larvae increased sharply between mid-April and the end of May in Carter Pond Outlet; prevalence of *H. melusinae* rose from 9 to 100% and species diversity increased from two to five species (Table 1, column 1 vs 4). Not all blackfly species had the same prevalence in a given sample; on April 14, preva-

lence of *H. melusinae* and *S. culicis* was considerably higher in *C. dacotensis* vs *S. vittatum* (column 1 vs 2); likewise, on August 8–9, *S. vittatum* lacked *G. homothallica* infection in Fish Hatchery Stream while 35% of the other *Simulium* spp. were infested (column 6 vs 7). During April 14–24, infestation in *C. dacotensis* differed between two locations in the same stream, i.e., Whittaker Brook and its origin, Carter Pond Outlet, located ca. 500 m upstream (column 2 vs 3); both prevalence of *H. melusinae* and trichomycete species diversity increased with distance downstream. In contrast, no clear trend or pattern was noted in prevalence or diversity of trichomycetes with distance downstream in a Colorado watershed (Lichtwardt and Williams, 1988).

An unusual pointed structure (Fig. 6), always located at the tip of a branch, was observed in the April–May samples in the hindguts of five larvae (one *C. dacotensis* from Whittaker Brook; one *S. venustum/verecundum* and three *S. vittatum* from Carter Pond Outlet). Typically only one of these structures (maximum two) was present in each of these five larvae. The thalli bearing these structures resembled *G. homothallica*, but positive identification was not possible due to lack of trichospores or visible holdfast. These pointed structures to some extent could be interpreted as developing type IV zygospores (Moss *et al.*, 1975). However, if the thalli truly were *G. homothallica*, a species only known to have type III zygospores, the most probable explanation would be that these structures were early aborted



zygospores. (For a detailed discussion of zygospore morphology, see Lichtwardt, 1986.)

In the laboratory infestation trials with *S. simulii*, developing thalli were observed in *S. vittatum* after 6 days and immature trichospore production after 8 days. Larvae in the low- and high-dosage beakers had infestation rates of 57% ($n = 7$) and 100% ($n = 20$), respectively. All control larvae lacked hindgut thalli. Laboratory infestation of blackflies with trichomycetes has not been previously reported. Our experiments with *S. simulii* demonstrated that infestation can be initiated in blackflies following exposure to *in vitro* produced trichospores and that trichospore production can commence within approximately 1 week at 14°C. The only other laboratory-induced infestations of aquatic larvae by axenic cultures of trichomycetes have used mosquitoes and trichospores of various species of *Smittium* (Chapman, 1966; Williams and Lichtwardt, 1972; Horn, 1989).

Although the *Simulium* flies were not identified to species in the 1987 data, the six infected flies from Carter Pond Outlet on May 21, 1993, were *S. venustum*/*verecundum* (damage to genitalia prevented further identification). Cysts removed from five of these flies and cultured on agar produced *H. melusinae* (Fig. 7) and *G. homothallica* (Fig. 8) trichospores. The *H. melusinae* trichospores produced from cysts possessed a membranous sheath (Fig. 7). Moss and Descals (1986) did not report any similar structure on their *H. melusinae* trichospores derived from cysts. Their cysts, however, were field collected in association with oviposited eggs, while ours were cultured in the laboratory from cysts directly removed from ovaries.

Prevalence of trichomycete cysts in adult blackflies varied considerably among locations and sampling dates (Table 2). During the last 10 days of May, infected flies were relatively common in Carter Pond Outlet samples in 1987, rare in 1993, and absent in 1995. While prevalences as high as 80% were recorded during these 3 years, 68% (13/19) of these adult samples had prevalences of 0–2%. The only other reports of trichomycete prevalence in ovipositing blackflies are from Newfoundland, Canada and also reflect varying infection rates. Yeboah *et al.* (1984) reported “phycomycete” infection in up to 3.7% in *Prosimulium mixtum* and 40% in *Stegopterna mutata* at the end of May and 4.2%

TABLE 2

Prevalence of Ovarian Cysts among Ovipositing Simuliidae in the Area of Cambridge, New York

Stream	Date of collection (month/day/year)	Prevalence	
		%	No. dissected
Carter Pond Outlet	5/20/87	38	32
	6/15/87	33	6
	6/18/87	0	12
	5/21/93	2	209
	5/23/93	0	168
	5/24/93	0	89
	4/24–5/11/95	0	207
	5/31/95	0	30
Jackson Reservoir Outlet	5/26/87	0	327
Lake Lauderdale Outlet	6/02/87	0	179
Thurber Pond Outlet	6/09/87	27	26
	6/18/87	80	10
	7/01/87	11	46
	7/14/87	2	56
	5/12/93	0	8
Cossayuna Pond Outlet	6/11/87	2	615
	6/25/87	19	58
	7/07/87	2	235
Flaxmill Outlet	7/29/87	0	88

in mid-June. Undeen and Nolan (1977) found 10.8% infection in *P. mixtum* in a May 17–20 sampling; Undeen (1979) observed over 50% infection in *S. mutata* from Newfoundland.

The factors determining trichomycete prevalence in adults are unclear. In 1995, despite extensive larval infestation with *H. melusinae* and *G. homothallica*, *C. dacotensis* and *S. vittatum* adult females did not appear parasitized by these trichomycetes. In Carter Pond Outlet on April 14, 29% of the *C. dacotensis* larvae contained *H. melusinae*; 500 m downstream of this outlet, i.e., in Whittaker Brook, *C. dacotensis* larvae sampled on April 24 had an 83% prevalence with *H. melusinae* and 75% with *G. homothallica* (Table 1). In contrast, however, *C. dacotensis* adults ($n = 207$) collected at Carter Pond Outlet from April 24 to May 11 were all uninfected. Likewise, *S. vittatum* larvae sampled in Carter Pond Outlet during the last week of

FIG. 2. *Stachylina* sp. thallus with developing trichospores attached to peritrophic membrane of *Simulium vittatum*.

FIG. 3. Released *Stachylina* sp. trichospore with a single basal appendage.

FIG. 4. A second species of *Stachylina* attached to the peritrophic membrane of a *Simulium vittatum* larva, with two trichospores remaining on the originally 4-spored thallus. The upper trichospore has just released from its generative cell and shows the basal appendage still unfurled.

FIG. 5. Developing *Smittium culicis* (?) trichospores; note the elongate shape and long collar.

FIG. 6. Unusual pointed structure found at the tip of a *Genistellopora homothallica* branch, possibly representing a malformed zygospore; see text.

FIG. 7. Coiled trichospores of *Harpella melusinae* produced in the laboratory from ovarian cysts of the fungus. Remnants of dark sheaths (arrow) in the background surrounded the cysts when they were removed from the ovaries.

FIG. 8. Loose trichospores of *Genistellopora homothallica* produced from germinated ovarian cysts. Note the very fine, almost invisible, multiple appendages on one released trichospore (arrow). Scale bars for Figs. 2–8, 20 μ m.

May had a 100% prevalence with *H. melusinae* and 35% with *G. homothallica* (Table 1), but *S. vittatum* adults ($n = 30$) sampled on May 31 had no cysts. Thus, the presence of trichomycetes in larvae does not guarantee overt infection in adults. Cyst production may prove to be affected by many factors, such as host species and environmental conditions (Lichtwardt, 1996).

The percentage of cysts that germinated and the time it took to produce mature trichospores in the laboratory varied, because different culture conditions were tried without standardization. Nonetheless, the following development occurred when cysts removed from ovaries were placed in distilled water on microscope slides. *H. melusinae* cysts began to produce germ tubes simultaneously from both ends of the oval cysts in less than 24 hr, and in 45–72 hr many trichospores had formed, matured, and released (Fig. 7). *G. homothallica* cyst development began slightly sooner. Initial germination from one end of the cysts was seen in 5–10 hr, trichospores began to form in 24–40 hr, and trichospores (Fig. 8) had matured and released within 60 hr.

While of great interest, resources did not permit conducting laboratory infection trials to establish that cysts from infected adults were capable of infesting blackfly larvae. Although highly probable, this route of infection has never been experimentally demonstrated (Fig. 1).

The first culture experiments were set up in water or on water agar without antibiotics. In cases where bacteria became numerous, one small drop of antibiotic stock solution was placed at the edge of the slide or on the surface of the agar away from the cysts. In subsequent experiments we incorporated antibiotics into the water and the agar medium before adding cysts. The temperatures at which these stages developed in the laboratory ($\approx 17^\circ\text{C}$) was approximately the temperature of the water at Carter Pond Outlet ($\approx 19^\circ\text{C}$) where the infected females would have "oviposited" the cysts.

Trichospores produced from the germinated cysts were identifiable by their typical shapes and number of basal appendages, but they were roughly 30–40% smaller in size than trichospores produced by thalli within larval hindguts. The most obvious explanation for the size difference is that cysts have more limited resources for trichospore production than do regular thalli. In fact, at the end of the developmental processes leading to trichospore formation from cysts, no protoplasm remains except in the trichospores.

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