Occurrence, Density, and Distribution of Parasitic Fungus *Hesperomyces virescens* (Laboulbeniales: Laboulbeniaceae) on Multicolored Asian Lady Beetle (Coleoptera: Coccinellidae)

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ABSTRACT The coccinellid-specific parasitic fungus *Hesperomyces virescens* Thaxter was found on the multicolored Asian lady beetle, *Harmonia axyridis* (Pallas), in fall and winter in Lebanon County, Pennsylvania. Research objectives were 1) to determine the density of *H. virescens* on field-collected *H. axyridis* adults held in the laboratory, and 2) to determine *H. virescens* presence, density, and distribution on *H. axyridis* adults in the field. In the laboratory, male and female *H. axyridis* adults hosted >150 *H. virescens* mature thalli (i.e., fruiting bodies); distributed primarily on the elytra and abdomen. At the overwintering site, *H. virescens* density per host was often <20 mature thalli, which were distributed primarily on the elytra of both sexes. On average, 52.5 and 57.4% of *H. axyridis* males and females, respectively, hosted *H. virescens* mature thalli in late winter (5 March 2003); <14% of either sex hosted mature thalli the following fall (15, 22, and 28 October and 10 November 2003) at the same site. This study suggests that *H. virescens* is an established parasite of *H. axyridis* in Pennsylvania but that field estimates of infection may vary considerably between dates that adult beetles arrive and depart from overwintering sites. Preponderance of fungal thalli on the dorsum rather than the ventrum of *H. axyridis* males suggests that mating behavior is not solely responsible for transmission of *H. virescens* from infected to noninfected adults.

KEY WORDS aggregations, overwinter, infection, *Harmonia axyridis*, Pennsylvania

LABOULBENIALES IS A LARGE ORDER of ascomycetous fungi containing almost 2,000 described species distributed throughout much of the world (Weir and Beakes 1995, Weir and Blackwell 2001). The majority of species have been called ectoparasites, obligate pathogens, or commensals of insects, mites, and millipedes (Steinhaus 1949, Tanada and Kaya 1993). Members of the family Laboulbeniaceae are biotrophic, which means that they survive only on the cells or tissues of living hosts (Richards and Smith 1954, Whisler 1968), the most common of which are adult beetles (Tavares 1985, Weir and Hammond 1997, Santamaria 2001). Infection occurs when the basal cell of a two-celled ascospore attaches to the cuticle of a susceptible insect. In Laboulbeniales species (e.g., *Hesperomyces virescens* Thaxter), a haustorium is produced from the basal cell that penetrates through the cuticle and may extend into the hemocoel and even into various tissues of the host (Tavares 1979, Weir and Beakes 1996). The cell of the ascospore undergoes repeated mitotic divisions to produce distinct parts of the determinate thallus. The thallus is composed of an array of cells that form the reproductive organs of the fungus (Tavares 1979).

Most species of Laboulbeniales cause little or no harm to their hosts (Whisler 1968, Weir and Beakes 1995). Some species may compete with their hosts for nutrients within the hemocoel, but normally there is enough nourishment for both (Tavares 1979). Some Laboulbeniales may reduce the life span and egg production of their hosts (Strandberg and Tucker 1974), reduce host mobility (Gemeno et al. 2004), or cause premature mortality when clusters of thalli on mouthparts and antennae impede feeding behavior (Bro Larsen 1952). *H. virescens* infected up to 95% of the adults of coccinellid *Chilocorus bipustulatus* L. in citrus groves in Israel, resulting in premature mortality of their hosts (Kamburov et al. 1967). However, others have suggested that lack of prey in the groves rather than infection by *H. virescens* was primarily responsible for the decline of *C. bipustulatus* populations (Applebaum et al. 1971).

Laboulbeniales species are often transmitted from one insect to another via sexual contact during mating or by nonmating physical contact while establishing overwintering aggregations (Benjamin and Shanor 1952, De Kesel 1995). Infection may reach a maximum

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in the fall at the onset of overwintering and in early spring when mating occurs before dispersal from sites (Weir and Beakes 1996). Very few species attack larval stages of insects, so infection of overwintering adults might be critical to persistence of Laboulbeniales populations in cool, temperate regions (De Kesel 1995). A less common route of transmission occurs when insects come in contact with ascospores while crawling through the soil (Lindroth 1948, Andersen and Skorping 1991). In addition, autoinfection can occur when the fungus spreads from the initial site of attachment to other parts of the body of the same host (Whisler 1968, Strandberg and Tucker 1974).

Incidence of infection can be influenced by host sex; certain species have been known to attack only males or females (Benjamin and Shanor 1952). Infection can be restricted to specific positions on the host (Thaxter 1896, Richards 1952, Benjamin and Shanor 1952, Richards and Smith 1955, Benjamin 1965). Thalli were more prevalent on the ventrum of males and the dorsum of female beetles (Benjamin and Shanor 1952, Whisler 1968, Andersen and Skorping 1991, Welch et al. 2001) and flies (Whisler 1968, Hedstrom 1994). *Laboulbeniales clivinalis* Thaxter thallus density was greatest during the mating season of its carabid host *Clivina fossor* (L.) (De Kesel 1995). Thallus distribution was affected by host sex but only during the mating season of *C. fossor* (De Kesel 1995).

The genus *Hesperomyces* Thaxter includes only five species; four parasitize coccinellids and one parasitizes mycetophagids (Tavares 1985). *H. virescens* represents the type species of the genus. It was first discovered on the coccinellid *Chilocorus stigma* (Say) (correct name for *Chilocorus bivulnerus* Mulsant) in California (Thaxter 1931). This parasite was recently discovered attacking *Harmonia axyridis* (Pallas), for the first time anywhere in the world, in vineyards and mung bean and alfalfa fields in Ohio in summer and fall 2002 (Garcés and Williams 2004). Other reported coccinellid hosts of *H. virescens* are *Hippodamia convergens* Guerin-Meneville in southeastern United States (Thaxter 1931), *C. bipustulatus* in Israel (Kamburov et al. 1967), *Psyllobora* (=*Thea*) sp. in France (Tavares 1985), *Adalia bipunctata* L. in southern Europe (Weir 1996), *Cycloneda sanguinea* (L.) in England (Tavares 1979), *Olla v-nigrum* (Mulsant) in Fiji (Weir and Beakes 1996), and *Eriopis connexa* Germar in Argentina (Thaxter 1931). It is apparent that *H. virescens* is a generalist parasite of lady beetles throughout the world.

H. axyridis is an entomophagous lady beetle originating in Asia. It was released in North America for classical biological control (Gordon 1985) and became firmly established in Louisiana and Mississippi (Chapin and Brou 1991) and then in the southeastern states (Gordon and Vandenberg 1991, Tedders and Schaefer 1994) and western states (Dreistadt et al. 1995, LaMana and Miller 1996). *H. axyridis* has expanded its range northward into most northern states and Canada (Day et al. 1994, Hoebeke and Wheeler 1996, McCorquodale 1998).

In spring and summer, *H. axyridis* larvae and adults are important predators of aphids and scales in apple, pecan, and citrus ecosystems (Tedders and Schaefer 1994, Brown and Miller 1998, Michaud 2002, Brown 2004). In autumn, adults migrate from feeding to overwintering sites. In eastern North America, the most obvious overwintering sites have been houses and other artificial structures (Nalepa et al. 1996, Schaefer 2003). This study reports the serendipitous discovery of *H. virescens* on multicolored Asian lady beetles in fall and winter at an overwintering site in Pennsylvania. Research objectives were 1) to determine the density of *H. virescens* on field-collected *H. axyridis* adults held under artificial conditions in the laboratory, and 2) to determine the occurrence, density, and distribution of *H. virescens* on *H. axyridis* in the field.

Materials and Methods

Overwintering Site and Collection of Beetles in Fall 2002. Beetles were observed and collected from a well-established overwintering site near Mt. Gretna, Lebanon County, Pennsylvania. The site was a cylindrical, reinforced steel and concrete observation tower (20.1 m in heigh, 4.6 m in diameter), consisting of five levels, including an entrance level and levels 1–4, ascending to the top of the tower. The tower is situated on the crest of a ridge (326-m elevation, 40° 14.78′ N, 76° 27.35′ W) in a small clearing surrounded by forests of hardwoods and a few conifers. Beetles have established overwintering aggregations on the inner walls (along the south-facing ladder-way) of levels 1–4 since fall 1993. The number of overwintering beetles found in tower levels 1–4 did not differ significantly, at least during 1995–1997 collections (Schaefer 2003).

On 20 November 2002, a few thousand aggregating *H. axyridis* adults were brushed from the inner walls of tower levels 2 and 3 into pint-sized (473.2-ml) ice cream paper containers and transported in an ice chest to the USDA–ARS, Beneficial Insects Introduction Research Laboratory (BIIRL), Newark, DE. Beetles were cold-stored (at ≈5°C) in a refrigerator for 21 d (until 11 December 2002) inside the same containers placed in at the time of collection.

Detection of Fungus and Thallus Density on Laboratory-Acclimatized Beetles in Communal Cages. A sample of 445 *H. axyridis* adults (of mixed sexes) from those cold-stored for 21 d at BIIRL was shipped overnight to the USDA–ARS, Biological Control and Mass Rearing Research Unit (BCMRRU), Mississippi State, MS, for experimentation. Specimens were removed from pint-sized containers on 12 December 2002 and approximately equal numbers were placed at random into one of two polypropylene cages (30 by 30 by 30 cm, 24 mesh size; Bug Dorm 1, MegaView Science Education Services Co., Ltd., Taiching, Taiwan), provisioned with food (pure honey) and sterile water on cotton wads at the base of each cage. Both cages contained mixed sexes of beetles of unknown age. Both cages were placed in a growth chamber (22°C, 60–65% RH, and a photoperiod of 12:12 [L:D] h).

On 18 December 2002, beetles were removed from the growth chamber to begin sexing. Sexually dimorphic characters can be seen on the last two abdominal sternites of *H. axyridis* females and males (E.W.R., unpublished observations). Using a stereo-zoom microscope, fungal thalli (i.e., fruiting bodies) were detected on the integument of some beetles. The number of *H. axyridis* adults with or without fungal thalli on their integument was determined on 18–19 December 2002. All adults (infected and noninfected) were kept within the same communal cages inside the growth chamber. Infected beetles were those individuals that had at least one mature thallus on the integument. This was a very conservative estimate of infection. However, morphological characters of mature thalli (rather than ascospores or developing thalli) are most often used to recognize Laboulbeniales species.

Eight infected beetles (four males, four females) were shipped to a mycologist (A. Weir, SUNY, Syracuse, NY) for identification. The fungus was identified as *H. virescens.* Voucher specimens of the fungus on *H. axyridis* adults are stored at the USDA–ARS, Biological Control of Pests Research Unit, Stoneville, MS, and the Department of Environmental and Forest Biology, State University of New York, Syracuse, NY. Immature and mature stages of *H. virescens* on other coccinellid hosts have been illustrated previously (Thaxter 1931, Kamburov et al. 1967, Tavares 1979, Weir and Beakes 1996, Christian 2001).

All beetles were resexed and rechecked for *H. virescens* thalli on 30 and 31 December 2002 and on 2 January 2003. On 9 January 2003, a random sample of 46 infected males and 46 infected females was removed from respective cages and preserved, individually, inside 2-ml plastic vials containing 70% ethanol (vol:vol). To estimate the extent of infection in the culture, the density of *H. virescens* mature (i.e., fully developed) thalli found on these infected beetles was determined in relation to host sex (male versus female) and body part (e.g., head, antennae, and pronotum). Each beetle served as a replicate. Each thallus represents a reproductive unit (Tavares 1979). A stereo-zoom microscope (10–90× magnification) was used for counting fungal thalli. Body parts (with code) were the following: antennae (An), head (He, including mouthparts, eyes, and palpi), pronotum (Pro), left elytron (El-L), right elytron (El-R), prothorax (Pt), meso- and metathorax combined (MMt), abdomen (Ab), right foreleg (1 R), left foreleg (1 L), right midleg (2 R), left midleg (2 L), right hindleg (3 R), and left hindleg (3 L).

Thallus Presence and Density on Beetles in Late Winter 2003. We determined the presence and density of *H. virescens* on field-collected *H. axyridis* adults that were not kept alive in the laboratory. The observation tower at Mt. Gretna, PA, was revisited on 5 March 2003. Large aggregations were not present on this date. Regardless, 206 *H. axyridis* adults (consisting of living and dead beetles) were all that could be found on the inner walls and floors of tower levels 2 and 3. Beetles from both tower levels were combined into the same pint-sized paper container. On the same day of collection, live beetles were separated from dead ones and placed within separate glass shell vials containing 70% ethanol at BIIRL.

Samples were shipped to BCMRRU, and all beetles were examined under a stereo-zoom microscope. Thalli on dead beetles were detectable after they had been rehydrated when placed in ethanol. Because beetles from either tower level were not kept separate upon collection, no treatment replication was achievable when determining the presence (or absence) of mature thalli on lady beetles. The total number of beetles with or without one or more mature thallus on their integument was noted in relation to host sex and host status (i.e., living versus dead at the time of collection). These data were generated from a total of 206 beetles (85 males, 121 females) collected on 5 March 2003. The number of mature thalli per beetle (i.e., thallus density) was determined. Each infected beetle served as a replicate. Thallus density was generated from 78 dead beetles (28 males, 50 females) and 39 living beetles (17 males, 22 females). Beetles were examined under a stereo-zoom microscope.

Infection Rate and Thallus Distribution on Beetles in Fall 2003. We estimated the percentage of beetles infected with *H. virescens* as they arrived at the observation tower in fall 2003 and determined the distribution of *H. virescens* on beetle body parts. (We speculated that infected rather than uninfected beetles would arrive at the overwintering site later in the flight period.) Beetles usually began arriving at the site in mid-October, and flight to the site usually continued until early to mid-November of each year (Schaefer 2003). On four collection dates (15, 22, and 28 October and 10 November 2003), all beetles found aggregating on the outer and inner walls of tower levels 2 and 3 were removed and placed into separate pint-sized paper containers. On the same day of collection, a random sample of adults (of mixed sexes) was removed from the containers and preserved in 70% ethanol at BIIRL. This data set represented 1,740 beetles (649 and 488 males from tower levels 2 and 3, respectively; and 289 and 314 females from levels 2 and 3, respectively). All other beetles were cold-stored (at ≈5°C).

Preserved beetles were shipped to BCMRRU for sexing, determining infection rate and thallus distribution. The percentage of infected beetles (i.e., infection rate) was estimated in relation to host sex and collection date. Thallus distribution was reported as the percentage of infected beetles with mature thalli distributed only on head, legs, elytra, abdomen, or combinations thereof. Recording the distribution of thalli on combined body parts (such as legs and abdomen, or legs and elytra) provided clues as to whether autoinfection—as beetles groomed themselves—was vital to spread of *H. virescens* to unexpected body parts of male and female beetles. A total of 121 infected beetles (91 males, 30 females) were representative of this test. Collection dates served as replicates for these percent data. Beetles were examined under a stereo-zoom microscope.

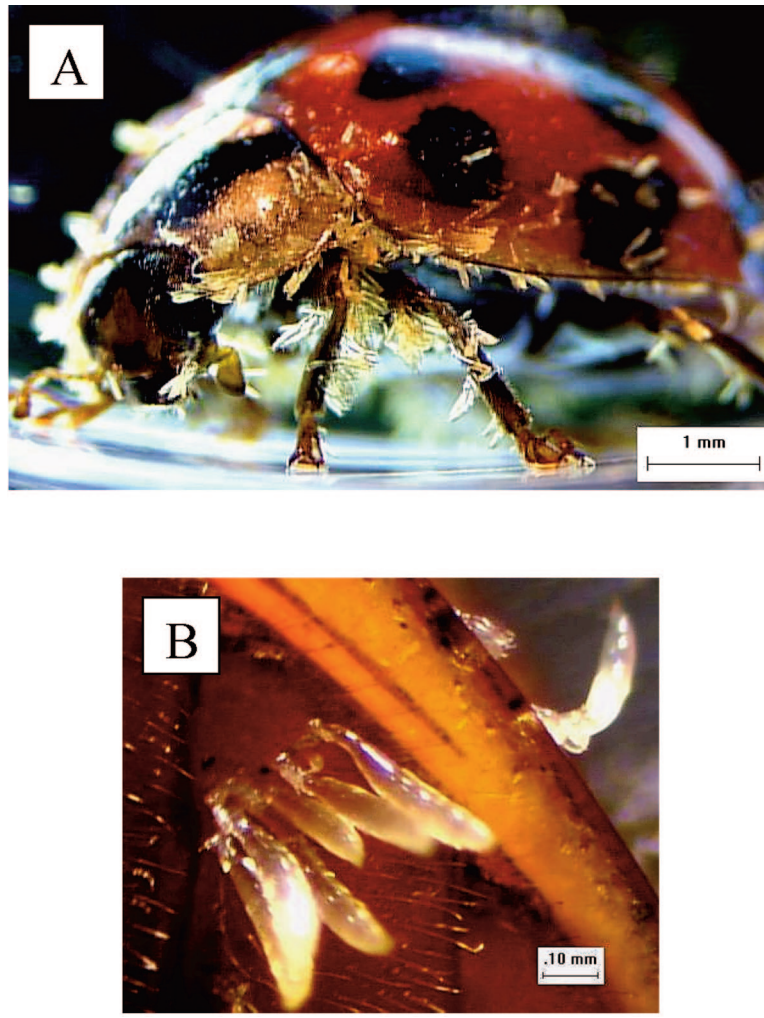

Fig. 1. (A) Distribution of *H. virescens* thalli on a *H. axyridis* female (at 20× magnification) that was removed from an overwintering aggregation (on 20 November 2002), cold-stored, and then maintained in the laboratory for nearly a month at 22°C. (B) Close-up view (110×) of a cluster of *H. virescens* thalli on abdominal sternites of another *H. axyridis* female subjected to the same conditions.

Data Analysis. An analysis of variance (ANOVA) was used to evaluate the significance of treatments (host sex, status, and body part) on thallus density in late fall 2002 and late winter 2003. A χ^2 analysis of three 2 by 2 contingency tables (with Yates correction for continuity) was performed to test for independence of thalli presence/absence on host sex, and status in late winter 2003. ANOVA was used to evaluate the significance of treatments (host sex and collection date) on infection rate and the significance of treatments (host sex and body part combinations) on thallus distribution in fall 2003. Data subjected to ANOVA were analyzed as a completely randomized design by using sex, status, body part, or collection date as factors, as indicated for respective experiments. Absolute data were square-root transformed (except for data subjected to χ^2 analysis), and percent data were arcsine-transformed before analysis and a Tukey's multiple comparison test was used for separation of means after ANOVA, when necessary (Zar 1999). Means were significantly different when $P < 0.05$. Statistical analyses were performed with SYSTAT (1998) or SigmaStat (2004) software. Only untransformed data are presented.

Results

Detection of Fungus and Thallus Density on Laboratory-Acclimatized Beetles. *H. virescens* was first detected on *H. axyridis* adults as they were being sexed. Twenty-two percent of adults (n = 445, combined sexes) were infected, as determined on 18–19 December 2002. The percentage of males versus females infested was not recorded on these dates. Because infected beetles were not separated from uninfected conspecifics in communal cages, by 2 January 2003,

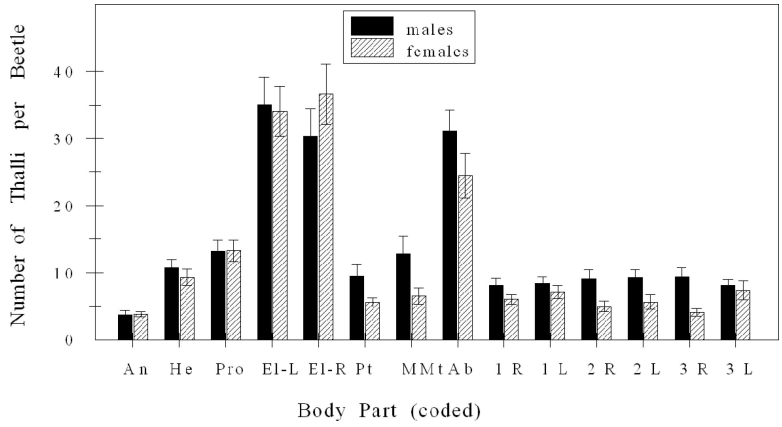

Fig. 2. Mean ± SEM number of *H. virescens* mature thalli per beetle per body part. *H. axyridis* males and females were collected in fall 2002 and maintained in the laboratory (as described in Fig. 1 legend). Body parts (with codes) included antennae (An), head (He), pronotum (Pro), left elytron (El-L), right elytron (El-R), prothorax (Pt), meso- and metathorax (MMt), abdomen (Ab), right foreleg (1 R), left foreleg (1 L), right midleg (2 R), left midleg (2 L), right hindleg (3 R), and left hindleg (3 L). Mature thalli on 46 male and 46 female infested beetles were counted.

100% of *H. axyridis* males ($n = 249$) and females ($n = 181$) had *H. virescens* thalli on their integument. Figure 1, A and B, illustrates *H. virescens* thalli on beetles that had been within the same cage for 28 d at 22°C. Detailed observations of the behavior of infected beetles were not made in this study. However, heavily infested beetles (i.e., those harboring 100 or more *H. virescens* mature thalli) were often seen using their legs to try to remove the fungus. Thus, autoinfection (i.e., spread of infection between body regions of the same host) was likely responsible for the presence of nearly 10 mature thalli on head and legs of male beetles in the laboratory cultures.

Host sex had a significant influence on thallus density per beetle ($F = 19.85$; df = 1, 1260; $P < 0.0001$); thalli were densest on males rather than females. The average total number of mature thalli per beetle was 199 and 169 for *H. axyridis* males and females, respectively. Host body part had a significant influence on thallus density ($F = 55.7$; df = 13, 1260; $P < 0.0001$); thalli were densest on elytra and abdomen than on any other body part of both sexes (Fig. 2). The interaction between sex and body part was not statistically significant ($F = 1.7$; df = 13, 1260; $P = 0.05$).

Thallus Presence and Density on Beetles in Late Winter 2003. Presence of *H. virescens* thalli on beetles collected on 5 March 2003 was dependent on status ($\chi^2 = 7.10$, df = 1, $P = 0.008$, $n = 206$); more dead than living beetles had one or more mature thallus on their integument. Upon closer inspection, thallus presence was dependent upon the status of females ($\chi^2 = 9.97$, df = 1, $P = 0.002$, $n = 121$); more dead than living females hosted *H. virescens* thalli (Fig. 3). Thallus presence was not dependent on male status ($\chi^2 = 0.05$, df = 1, $P = 0.82$, $n = 85$). Note that 54.9 and 72.5% of dead males and females, respectively, were infected;

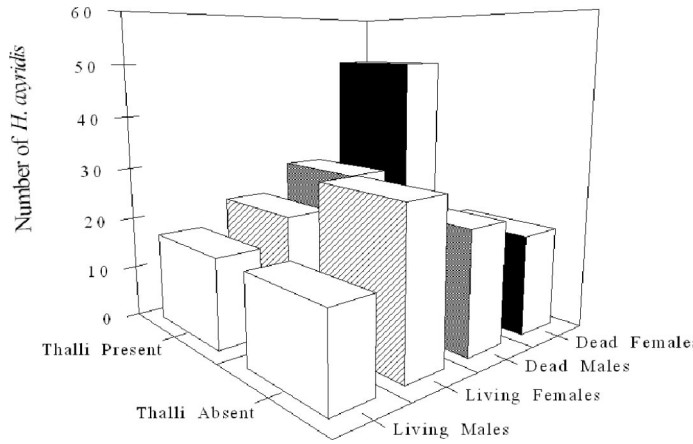

Fig. 3. Number of *H. axyridis* males and females (living versus dead) infected with *H. virescens* at the overwintering site in late winter 2003. These data were generated from a total of 206 beetles (85 males, 121 females) collected on 5 March 2003. These data were subjected to χ^2 analysis in this test.

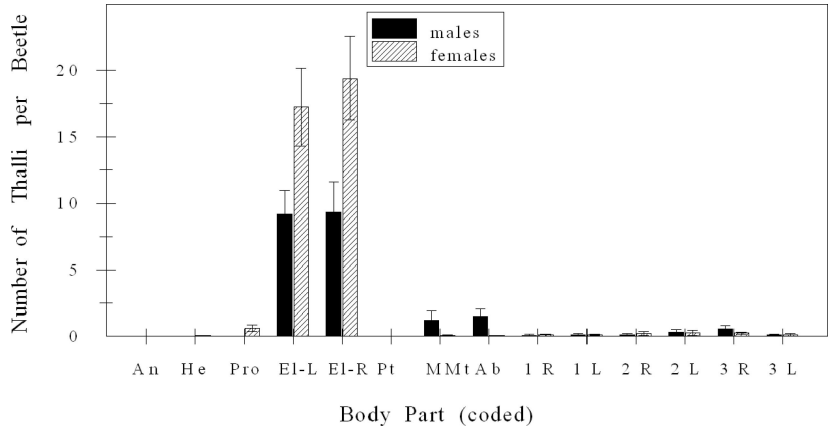

Fig. 4. Mean ± SEM number of *H. virescens* mature thalli per beetle per body part. *H. axyridis* males and females were collected at the overwintering site in late winter (5 March) 2003. Body parts (with codes) were the same as described in Fig. 2 legend. Thallus density was determined from 78 dead beetles (28 males, 50 females) and 39 live beetles (17 males, 22 females). Only two factors (sex and body part) were illustrated in this figure.

50.0 and 43.3% living males and females, respectively, were infected in late winter 2003. Regardless of status, an average of 52.5 and 57.4% of *H. axyridis* males and females, respectively, were infected by *H. virescens.* Thallus presence was not dependent on sex of living beetles ($\chi^2 = 0.23$, df = 1, $P = 0.63$, $n = 86$) or dead beetles ($\chi^2 = 3.24$, df = 1, $P = 0.07$, $n = 120$).

Thallus density was influenced by host body part but not by host sex or status (body part: $F = 85.3$; df = 13, 1512; $P < 0.0001$; sex: $F = 1.4$; df = 1, 1512; $P = 0.2$; and status: $F = 1.2$; df = 1, 1512; $P = 0.3$). Density was greatest on left and right elytron rather than mesothorax and metathorax, abdomen, or any other body part (Fig. 4). The interaction between sex and body part was significant ($F = 3.1$; df = 13, 1512; $P < 0.0001$); thallus density was slightly greater on the elytra of females than males. On average, females had practically none and males had a few mature thalli on the mesothorax, metathorax, and abdomen. Two-way interactions between treatments were not significant (sex and status: $F = 0.9$; df = 1, 1512; $P = 0.3$; and body part and status: $F = 1.2$, df = 13, 1512; $P = 0.2$) and the three-way interaction between treatments was not significant ($F = 1.2$; df = 13, 1512; $P = 0.9$).

Infection Rate and Thallus Distribution on Beetles in Fall 2003. Less than 14% of H. *axyridis* adults had *H. virescens* mature thalli anywhere on their integument at any collection date (Fig. 5). Infection rate was neither influenced significantly by collection date ($F = 0.78$; df = 3, 8; $P = 0.53$) nor host sex ($F = 4.10$; df = 1, 8; $P = 0.08$), and the interaction between date and sex was not significant ($F = 0.12$; df = 3, 8; $P = 0.9$). Males were more abundant than females on each collection date. The average percentage of males (out of the total number of beetles in this data set) per date was 64, 57, 65, and 76% on 15, 22, and 28 October and 10 November 2003, respectively.

H. virescens thalli were distributed primarily on the elytra rather than on the head, legs, abdomen, or any combinations thereof, of *H. axyridis* adults ($F = 31.8$; df = 12, 78; $P < 0.0001$; Fig. 6). The interaction between body part combination and sex was significant ($F = 2.83$; df = 12, 78; $P = 0.003$); a greater percentage of *H. axyridis* females rather than males had thalli only on the elytra. Host sex, when considered independently, had a marginally significant influence on thallus distribution ($F = 4.02$; df = 1, 78; $P = 0.048$). Very few *H. axyridis* males and no females had thalli only on legs, head and legs, legs and abdomen, and any of the three or four body part combinations of head, legs, elytra, and abdomen (Fig. 6).

Discussion

Host–Parasite Associations. This study suggests that *H. virescens* is an established parasite of *H. axyridis* in Pennsylvania. It was first discovered in Ohio on *H. axyridis* in vineyards, mung bean, and alfalfa fields in summer and fall 2002 (Garcés and Williams 2004). *H. axyridis* was first detected in Pennsylvania in 1993

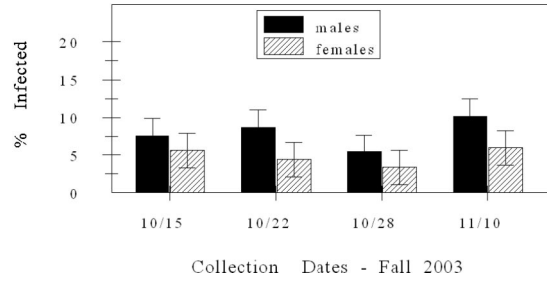

Fig. 5. Mean ± SEM percentage of *H. axyridis* males and females infected with *H. virescens* at the overwintering site in fall 2003. This data set represented 1,740 beetles (649 and 488 males from tower levels 2 and 3, respectively; and 289 and 314 females from levels 2 and 3, respectively), which had been collected "alive" at the site and then preserved in ethanol. This experiment consisted of four collection dates; 15, 22, and 28 October 2003 and 10 November 2003.

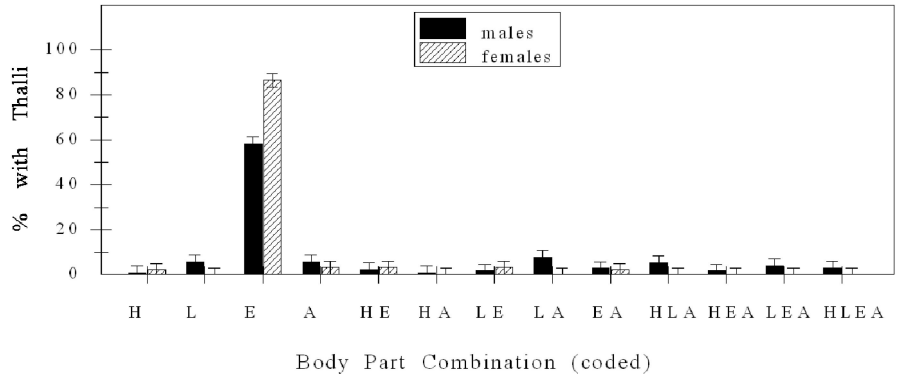

Fig. 6. Mean ± SEM percentage of infected *H. axyridis* with *H. virescens* thalli on body parts, and combinations thereof, in fall 2003. A total of 121 infected beetles (91 males, 30 females) were representative of this test. Only beetles "alive" at the time of collection were included in this test. Four collection dates were involved in this experiment (15, 22, and 28 October 2003 and 10 November 2003). Data represented *H. axyridis* adults with mature thalli distributed only on their head (incl. mouthparts and antennae), legs, elytra, abdominal sternites, or combinations thereof, per collection date. Body part combinations (with codes) included head (H), legs (L), elytra (E), abdominal sternites (A), head and elytra (HE), head and abdominal sternites (HA), legs and elytra (LE), legs and abdominal sternites (LA), elytra and abdominal sternites (EA), head and legs & abdominal sternites (HLA), head and elytra and abdominal sternites (HEA), legs and elytra and abdominal sternites (LEA), and head and legs and elytra and abdominal sternites (HLEA).

(Day et al. 1994). It is unlikely that *H. virescens* could have gone unnoticed on *H. axyridis* for nearly a decade, considering the attention that this beetle has received in recent years. Could physical contact between coccinellid species precipitate the transfer of *H. virescens* ascospores from an established to a novel host? This question is not unreasonable, because the current distribution of *H. axyridis* overlaps with *H. convergens*, *A. bipunctata*, *C. sanguinea*, and *O. v-nigrum* (Gordon 1985), all of which have served as hosts of *H. virescens*. However, there are no published records of *H. virescens* on any coccinellid in North America, except for *H. axyridis*, since 1931 (see Introduction). More research will be required before the origin of the *H. axyridis–H. virescens* association can be ascertained.

Fungus Spread and Thallus Density on Hosts in the Laboratory. Infection of 100% of *H. axyridis* adults when confined in crowded cages for up to 28 d (at 22°C) demonstrates the propagation potential of this fungus. Mating between *H. axyridis* males and females was rarely observed, so nonsexual contact between both sexes while feeding or while crawling in the cages may have been responsible for rapid spread of infection. Alternatively, beetles of both sexes may have become infected when contacting ascospores (released from mature thalli) on the bottom of communal cages. Ascospores of some species can survive for a few weeks in moist soil (Lindroth 1948). The importance of substrate infection is minor in relation to direct infection via contact between adult beetles (De Kesel 1993). It is also conceivable that *H. virescens* ascospores were not detected on some of the beetles at the time of collection. Removing beetles from cold storage and providing suitable temperature and humidity conditions could have promulgated the development of *H. virescens* immature stages within a month in the laboratory. These results also suggest that the presence of the parasite might interfere, in some situations, with the storage and commercial shipment of *H. axyridis* for biological control of pests. Methods for controlling *H. virescens* infection in laboratory cultures might include 1) adding a fungicide to the food provided to lady beetles (Gemeno et al. 2004), 2) decreasing beetle density per cage, and 3) cleaning cages more frequently.

Other Laboulbeniales species are known to transmit rapidly to new hosts under crowded conditions. For example, *Filariomyces forficulae* Shanor spread from infected to uninfected earwigs, *Labidura riparia* (Pallas), in laboratory arenas and developed from ascospore to mature perithecium (i.e., thalli) within 2 wk at 24°C (Strandberg and Tucker 1974). *Stigmatomyces ceratophorus* Whisler spread from infected to uninfected muscid flies, *Fannia canicularis* (L.), in the laboratory (Whisler 1968); mature thalli were dense on adult flies within 20–30 d. Male flies were more active than females and nonsexual contact between infected and uninfected males accounted for as much fungal spread as sexual contact between males and females (Whisler 1968).

The observation that thallus density was greatest on the elytra and abdomen than on any other body part was most likely due to differences in surface area between body parts and restricted growth of *H. virescens* on legs and venter rather than any *H. virescens* site specificity. (Note that site specificity has been reported for several Laboulbeniales species [Benjamin and Shanor 1952, Shanor 1955], and differences in physiological/chemical composition of the cuticle of various body parts on the same host (Richards 1952, Tavares 1979) may affect establishment of ascospores or successful maturation of thalli.) The elytra and abdomen might provide ample space for attachment of numerous *H. virescens* ascospores. However, preponderance of thalli on elytra and abdomen may sim-

ply represent areas of frequent contact between conspecifics. Richards and Smith (1955) observed mature thalli of *Herpomyces stylopygae* Spegazzini on the antennae of the oriental cockroach, *Blatta orientalis* L. (Blattidae), and rarely found them anywhere else. Infection was usually spread via contact and males were more often infected than females (Richards and Smith 1955).

Although the interaction between host sex and body part was insignificant in this study, the data revealed a possible trend. *H axyridis* males rather than females may have hosted a few more thalli on legs, mesothorax, metathorax, and abdomen. The fact that heavily infested beetles were often seen using their legs to try to remove the fungus suggests that autoinfection (i.e., spread of infection between body parts of the same host) was partly responsible for the occurrence of nearly 10 mature thalli on head and all legs of male beetles in the laboratory cultures (Fig. 2).

Fungus Spread, Thallus Density, and Distribution on Hosts in the Field. The observation that >50% of *H. axyridis* males and females (including living and dead beetles), collected in late winter, were infected suggests that *H. virescens* ascospores germinate and develop equitably on males and females under natural conditions. Sex specificity has been reported for several *Laboulbenia* species infecting adults of the carabid *Bembidion picipes* Kirby (Benjamin and Shanor 1952); *Laboulbenia perpendicularis* Thaxter thalli were found only on females, whereas *Laboulbenia truncata* Thaxter thalli were only on male beetles. Whisler (1968) stated that sex specificity was not characteristic of most species of Laboulbeniales.

An infection rate of 43–50% for living *H. axyridis* females and males collected in the field, respectively, in late winter, in this study, was higher than reported previously for other host species. Weir and Beakes (1996) found that 24% of *A. bipunctata* adults ($n = 70$) overwintering in a building in Europe were infected with *H. virescens.* Christian (2001) indicated that 29% of *A. bipunctata* adults ($n = 14$), found under tree bark during late winter and on bushes in summer, in Austria, were infected with *H. virescens.* The proportion of males and females infected with this fungus was not reported in either study.

The observation that the presence/absence of *H. virescens* thalli on *H. axyridis* adults at the overwintering site in late winter 2003 was dependent on host status was not expected. The fact that more dead than living females contained *H. virescens* thalli could mean that dead females were older and therefore had more time of exposure to infection when living than those females collected alive at the site. *H. virescens* and other Laboulbeniaceae are known to survive only on living hosts (Richards and Smith 1954, Whisler 1968). *H. axyridis* adults can live up to 3 yr (see Savoïskaya 1970 in Nalepa et al. 1996). Undoubtedly, differential mortality of *H. axyridis* at the overwintering site may have been attributed to other factors, such as desiccation, depletion of fat reserves, parasites, or pathogens (Lipa et al. 1975, Mills 1981, Leather et al. 1993, Webberley and Hurst 2002).

Older rather than younger generation carabids can host high densities of fungal thalli (De Kesel 1993). Differential mortality of older beetles may result in loss of the majority of fungal thalli, in terms of the entire host population, over the winter season. Winter is a critical period for survival of *L. clivinalis* because low temperature reduces fungal reproduction and therefore density of mature thalli per beetle (De Kesel 1995). Ascospore transmission between *H. axyridis* adults of different generations at overwintering sites might be vital to maintaining stable populations of the fungus from year to year.

The observation that host status had no effect on thallus density suggests that *H. axyridis* males and females are suitable hosts for *H. virescens* in the field and some mature thalli remain intact on the host (at least on the dorsum) during the overwintering period, even if the host expires. An average thallus density of <20 versus >100 on *H. axyridis* adults in the field versus laboratory, respectively, may reveal that opportunities for growth and development of *H. virescens* at such a magnitude, as seen in the laboratory, are probably infrequent in overwintering aggregations. Warm and humid conditions support the growth and development of Laboulbeniales species on aggregating hosts in the field (Meijer 1975, De Kesel 1995, Santamaria 2001).

The prevalence of *H. virescens* thalli on the dorsum and ventrum of *A. bipunctata* females and males, respectively, reflected the position of males on top of females during copulation (Welch et al. 2001). From a sample of *A. bipunctata* males ($n = 36$) collected in June 1999 in central London, 75% of them had *H. virescens* thalli on the ventrum only, 16.7% had thalli on the dorsum only, and 8.3% had thalli on both the ventrum and dorsum. *H. virescens* on *A. bipunctata* females ($n = 23$) from the same sampling period and location had a markedly different pattern of thallus distribution; 69.6% of female hosts had thalli on the dorsum only, 8.7% had thalli on the ventrum only, and 21.7% had thalli on both dorsum and ventrum (Welch et al. 2001).

Although *H. axyridis* probably mate in autumn before establishing aggregations and in spring before dispersing from overwintering sites (Nalepa et al. 1996), transmission of *H. virescens* via host sexual behavior does not account for the density and distribution of *H. virescens* thalli on the dorsum rather than the ventrum of *H. axyridis* males in late winter 2003 and again in fall 2003 in this study (Figs. 4 and 6). A logical explanation could be that males are simply more active in and around the aggregations. Perhaps, infected *H. axyridis* males climb on top of other males within the aggregations; thereby causing the expulsion of ascospores (out of mature thalli on the ventrum) onto the dorsum of other males. Garcés and Williams (2004) stated that *H. virescens* infection was concentrated on the ventroposterior of *H. axyridis* males and the dorsoposterior of females; but in late fall, after a period of aggregation, infection was more noticeable on the elytra, prothorax, and legs. Whether host sex had any influence on thallus density was not revealed by

Garcés and Williams (2004). Autoinfection (as a consequence of using the legs to groom or clean) probably contributed to only a very small proportion of the total thalli on the dorsum of males in this study. For example, mature thalli were present on legs and elytra of only a small percentage (10% or less) of male beetles (Fig. 6).

The fact that the presence of mature thalli on *H. axyridis* was not influenced by sex or collection date in fall 2003 suggests that 1) *H. axyridis* females are just as susceptible as males to infection, 2) both sexes can harbor *H. virescens* thalli before arrival at overwintering sites, and 3) infection rate has no apparent impact on flight behavior in the fall. The fact that fewer than 14% of *H. axyridis* adults hosted thalli in fall 2003 suggests that *H. virescens* abundance can vary considerably from one season to the next. Perhaps, contact between infected and uninfected *H. axyridis* adults in overwintering aggregations during unusually mild weather (in late fall and late winter) may account for the greater density of *H. virescens* on their hosts in late winter 2003 than in fall 2003. Garcés and Williams (2004) found that infection of *H. axyridis* males and females in July through August 2002 was 11 and 23%, respectively, but infection of males and females in October through November 2002 was 75 and 20%, respectively; males were more abundant than females in fall collections.

In conclusion, *H. virescens* density per host was much greater in the laboratory than at the overwintering site. Predominance of mature thalli on the dorsum rather than the ventrum of *H. axyridis* males in the field suggest that behaviors other than mating contribute to the distribution of *H. virescens* on *H. axyridis* in fall and winter. Nevertheless, physical contact between *H. axyridis* adults at overwintering sites provide opportunities for spread and persistence of *H. virescens* from year to year.

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