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Dissecting the association between a gall midge, *Asteromyia carbonifera*, and its symbiotic fungus, *Botryosphaeria dothidea*

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Abstract

The Ambrosia gall midge [*Asteromyia carbonifera* (Osten Sacken) (Diptera: Cecidomyiidae: Alycaulini)] consists, in part, of a complex of genetically differentiated populations that have diverged in gall morphology on the host plant *Solidago altissima* L. (Asteraceae). This divergence appears to be an incipient adaptive radiation that may be driven by parasitoid pressure. Understanding the mechanisms driving this genetic and phenotypic diversification requires a close examination of the relationship between the midge and its fungal associate *Botryosphaeria dothidea* (Moug.) Ces. & De Not. (Ascomycota: Dothideomycetes), whose mycelia actually form the protective gall structure. We used manipulative experiments to test the degree of interdependency of the fungus and the midge, and we employed field and laboratory studies to gain insight into the source of fungal conidia, which our data and observations indicate are collected by females and stored in specialized pockets (mycangia) on the ovipositor. Manipulative experiments demonstrate that fungal proliferation on the host plant is dependent on the midge larvae and larvae exhibit significant growth on the fungus alone. Field observations and experiments were unable to identify the source of mycangial conidia; however, analyses of conidia shape suggest a biotrophic source. We conclude that this association is an obligatory mutualism with respect to successful gall formation. These findings corroborate recent findings that the primary food source of the midge is the gall fungus.

Introduction

Mutualistic associations with microbes have likely played an important role in the phenomenal evolutionary and ecological success of the Insecta (Moran, 2002; Janson et al., 2008). Virtually every insect species that has been examined closely has been found to be engaged in some form of microbial mutualism, most frequently in the form of gut-associated bacteria (e.g., Buchner, 1965; Douglas, 1998). However, insect-fungal symbioses are also widespread across many insect groups including beetles (Scolytinae: Bentz & Six, 2006), ants (Formicidae; Mikheyev et al., 2006), moths (Tortricidae: Fermaud & Lemenn, 1989), and flies (Cecidomyiidae: Borkent & Bissett, 1985; Gagné, 1989; Anthomyiidae: Schiestl et al., 2006). In these associations, the insect typically benefits from using the fungus as a food source (leafcutter ants: Cherrett et al., 1989; gall midges: Bissett & Borkent, 1988; Ambrosia beetles: Farrell et al., 2001) in exchange for dispersing the fungus or promoting fungal outcrossing (Schiestl et al., 2006).

One of the most diverse and widespread groups of insects known to engage in symbiotic associations with fungi are the 'Ambrosia' gall midges, which represent a significant portion of the family Cecidomyiidae. The galls that these midges induce on their host plants are typically lined internally with fungal hyphae, which the developing larva(e) may feed upon (Haridass, 1987; Bissett & Borkent, 1988). Borkent & Bissett (1985) have provided tantalizing morphological evidence that at least some of these species actively transport fungi in specialized pockets, or mycangia, associated with the terminal abdominal segments. However, the nature of the Ambrosia midge-fungus interaction is largely speculative and there has been little

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experimental analysis of the association and the degree to which it represents an obligate mutualism. In fact, it has been argued that at least in some cases the fungi may represent opportunistic colonization rather than a strict mutualism. This controversy has been thoroughly reviewed (Haridass, 1987; Rohfritsch, 2008; Adair et al., 2009). Here, we employ observational and manipulative experiments to dissect the association between the gall midge *Asteromyia carbonifera* (Osten Sacken) (Diptera: Cecidomyiidae: Alycaulini) and its associated fungus *Botryosphaeria dothidea* (Moug.) Ces. & De Not. (Ascomycota: Dothideomycetes) in order to classify this symbiosis and understand its consequences for the ecology and evolution of the gall midge.

The galls induced by *A. carbonifera* and its associated fungus on host plants in the genus *Solidago* (goldenrod; Asteraceae) were observed over a century ago (Trelease, 1884; Batra, 1964). However, despite the efforts of several researchers (e.g., Batra, 1964; Gagné, 1968; Weis, 1982a,b; Bissett & Borkent, 1988), the biology of *A. carbonifera* is still poorly understood. Populations of *A. carbonifera* use a wide range of goldenrod species as hosts (Gagné, 1968), and preliminary analysis indicates that many of these populations exhibit host associated genetic differentiation (Stireman et al., 2010). Furthermore, within the single host plant *Solidago altissima* L., at least four genetically distinct gall morphotypes coexist, suggesting that *A. carbonifera* is adaptively radiating on this host (Crego et al., 1990; Stireman et al., 2008).

Understanding the details of the interaction between *A. carbonifera* and its fungal associate is likely key to understanding the adaptive diversification in this system. Borkent & Bissett (1985) mentioned that *A. carbonifera* transport fungal conidia, but only supplied photographs of the mycangia of *A. tumifica* and related species. Gagné (1968) attempted to initiate *A. carbonifera* galls in screen cages. Galls developed in his experiment, but we suspect that there were eggs already on the plants before they were caged. In fact, Gagné (1968, p. 13) does not reject this possibility when he states, 'It is possible also that…some newly laid eggs were overlooked.' Weis (1982a,b) clearly illustrated that larvae are variously protected from parasitoid attack by the fungal stroma and *A. carbonifera* has never been found in plant tissue not associated with the fungus (Trelease, 1884; Batra, 1964); nevertheless, some still question whether this association represents a mutualism. Therefore, the main goal of this study was to investigate the nature of this intimate association between the midge and the fungus. That is, is it truly a mutualism? If so, is it an obligatory relationship?

In particular, we were interested in (1) verifying earlier claims that adult females harbour fungal conidia in specialized structures on their ovipositor called mycangia and whether these conidia are present on eggs, (2) determining whether the midge is necessary for fungal growth and vice versa, (3) initiating galls under controlled conditions, (4) understanding where midges mate and obtain fungal conidia, and (5) determining the time required for fungus to appear on the top of the leaf after oviposition has occurred.

Study system

Asteromyia carbonifera is involved in complex interactions involving many interacting species. The four most prominent players are goldenrod (*Solidago* spp.), the gall midge *A. carbonifera*, the fungus *B. dothidea* (Bissett & Borkent, 1988; Janson et al., in press), and at least nine associated parasitoids, predators, and inquilines. Together, the fungus and midge larva produce at least four morphologically distinct galls on the leaves of *S. altissima.* Crego et al. (1990) described these four gall morphotypes on *S. altissima*. These were named crescents, flats, irregulars, and cushions in accordance with their overall morphology. Stireman et al. (2008) provided photographs and descriptions of these morphotypes. These galls are not deformations of plant tissue, but rather a gall formed mainly of fungus. Aside from chlorosis, the gall causes very little physical change at the cellular level of the plant (Camp, 1981). Other *Asteromyia* spp. also form similar Ambrosia galls on related host plants (Gagné, 1968, 1989; Stireman et al., 2010) and many *Asphondylia* spp. (Diptera: Cecidomyiidae) harbour the same fungal species (Bissett & Borkent, 1988; Adair et al., 2009); therefore, understanding this symbiosis in *A. carbonifera* may provide insight concerning other potential adaptive radiations.

Materials and methods

Biology and life history traits

Unless otherwise specified, the biology and life history traits were generally inferred from field observations at all the field sites (Table 1) over the summers of 2007–2009. To investigate the time for fungus to appear on the top of the leaves, *A. carbonifera* egg clutches were located on the most susceptible *S. altissima* clone in a common garden and marked (see subsequent section for details on the common garden). These clutches were checked daily until at least one position within the clutch began to show fungal growth on the top of the leaf (i.e., when the growth was about 0.3 mm in diameter). The leaf the eggs were found on was given an estimate of age in days between 0 and 3. The plants grow about 1 cm per day and the distance between pairs of leaves is roughly 1 cm at the top of the plant. Age 0 leaves were on the outside of the whorl and

Table 1 List of study sites with abbreviations, names, and coordinates[1]

Site abbreviation	Site name (all USA)	Latitude (N)	Longitude (W)
BCWMA	Beavercreek Wildlife Management Area, OH	39°45′59.09″	84°00′15.95″
HS	Huffman Metropark, Dayton, OH	39°48′28.30″	84°05′33.94″
KWP	Koogler Wetland Preserve, Dayton, OH	39°45′57.97″	84°00′40.96″
VS	Varner Rd., Dayton, OH	39°46′01.45″	84°00′56.51″
WSU-1	WSU Common Garden, Dayton, OH	39°47′14.96″	84°03′08.59″
WSU-2	WSU Services Site, Dayton, OH	39°47′25.12″	84°03′10.85″
SC-1	Millbrook Marsh, State College, PA	40°49′00.44″	77°50′04.13″
SC-2	Stewart Drive, State College, PA	40°49′50.16″	77°47′44.52″

[1]Coordinates obtained from Google Earth (Version 4.3).

had not dropped to a horizontal position. Age 1 leaves were still connected to the whorl, but were horizontal. Age 2 and 3 leaves were one and two nodes lower than the base of the whorl, respectively. Eggs were only found on age 0–3 leaves, but only the new growth was searched. Females were observed laying eggs in three instances in the field and these eggs were tracked as above. These clutches were followed until they formed mature, identifiable morphotypes (clutch sample sizes per morph: cushion: 22, flat: 4, irregular: 13, crescents: 0, and unknown: 1). A linear model with leaf age as a covariate was used to test for differences between the morphotypes in the time for fungus to appear on the top of the leaf.

Nature of the midge-fungus interaction

Two types of experiments were conducted to test the interdependency of the midge larva and fungus. The first experiment tested whether the growth of the gall fungus depends on the presence of the midge larva by removing larvae from very young galls and assessing fungal growth. The second experiment tested whether the midge larva feeds on the fungus by isolating the larva and fungus on growth media and tracking larval growth.

To test whether gall fungal growth is dependent on the presence of the midge larva, the larvae from very young galls were removed and fungal growth measured. *Solidago altissima* stems with very young blister galls (<2 mm in diameter) were collected from the Beavercreek Wildlife Management Area (BCWMA) site (Table 1). The stems were re-cut under water in the field and immediately placed in a container of cut-plant solution (Aquaplus; Syndicate Sales, Kokomo, IN, USA). On sunny days stems were placed in the shade during collection. The galls on the stems were randomly processed in the laboratory as negative controls, mocks, or removals. Controls were left untouched and intact. Mock removals ('mocks') controlled for possible confounding effects of the removal process; galls were dissected from the bottom of the leaf to the removal point and the larva was either touched with the forceps as if it was going to be removed or not touched. The removal treatment was conducted in the same manner as the mock treatment, but the larva was permanently removed from the gall. In both mocks and removals the disturbed fungal layer and leaf tissue was carefully placed back in its original position. These treatments were repeated in five independent experiments with a balanced sample size within each experiment (n = 6–9 per treatment). All galls were photographed with identical camera and magnification settings at the time of processing and after 10 days of incubation at room temperature. Fungal growth before and after incubation was measured in pixels in Photoshop (version 9.0; Adobe Systems Inc., San Jose, CA, USA), converted to net fungal growth per day, and standardized to the number of larvae present in the final gall. Standardization was necessary because during the initial stages of gall development each larva initiates a separate tiny gall. If the initial galls are close enough they will eventually merge leading to the appearance of a single large gall. In some cases immature galls processed as negative controls had neighbouring galls close enough that they eventually merged leading to the need to standardize net fungal growth to the number of larvae present.

To test whether the midge larva feeds on the fungus, immature galls were collected from the BCWMA site, dissected, and a small portion (ca. 10 mm^2) of black fungal stroma was transferred to malt-extract agar plates along with a young larva (n = 18), which was placed atop the portion of stroma. Negative controls without fungal transfer were also included (n = 17). The length of larvae was measured with an ocular micrometre mounted on a Nikon SMZ1000 stereomicroscope (Nikon Instruments, Melville, NY, USA) on the day of transfer and after 2 weeks of incubation at room temperature.

Preliminary experiments had shown that larvae grew significantly larger when transferred with the fungus as above, but the growth was marginal (i.e., a mean increase over the control of only 51 μm). Furthermore, in preliminary experiments most of the control plates were

also contaminated with gall fungus. Therefore, about 5 mg of medical grade Nystatin ointment (a fungicide, 100 000 USP g^{-1}; E. Fougera & Co, Melville, NY, USA) was applied to a 1-cm^2 area of both the treatment and control plates. The larva and fungus or larva alone (in the case of controls) was transferred atop this paste. The paste completely prevented fungal contamination in the controls and substantially reduced fungal growth in the treatments. The paste did not appear to affect the behaviour or survival of the transferred larvae.

A linear model in R (version 2.8.1; R Development Core Team, 2007) with 'larval end length' as the response and 'start length' as a covariate was used to determine fungal treatment effects. The full model included the following explanatory variables: larval start length, fungal treatment (gall fungus added or not), and their interaction.

Asteromyia eclosion behaviour

To understand the reproductive behaviour and to gain insight into where and how the adults obtain fungal conidia, mature irregular galls on *S. altissima* (SC-2 site; Table 1) and *rugosa* galls on *Solidago rugosa* P. Mill. (SC-1 site; Table 1) were marked and monitored daily in the field from 10 July to 7 August 2007 (see Gagné, 1968, for a description of *S. rugosa*-type galls). As adults emerged from the galls, their behaviour was recorded until they either flew off or otherwise became unobservable. On average, they were observed for 2.5 h. Ethograms were generated from these data and the relative frequency of the transition between behaviours calculated by dividing the number of times a transition from behaviour x to y occurred by the total number of behavioural transitions.

Conidia morphology

To gain insight into where fungal conidia are obtained, we compared the shape of egg-associated conidia to conidia obtained from other sources. Eggs of presumably different morphotypes were collected from the BCWMA and Koogler Wetland Preserve (KWP) sites (Table 1), mounted on microscope slides in EMD^{TM} lactophenol cotton blue (Fisher Scientific, Pittsburgh, PA, USA), and photographed at 400× with a Nikon Coolpix 8800 VR camera mounted on a Nikon Optiphot compound microscope (Nikon Instruments). The width and length of the conidia were measured from photographs in ImageJ (version 1.39u; National Institute of Health, Bethesda, MD, USA) after calibration with a photograph of a stage micrometre (2 mm, ruled to 0.01 mm; Micromaster, Fisher Scientific, Hampton, NH, USA). The length and width of these egg-associated conidia were compared to conidia from six other sources: (1) gall-isolated fungus grown at room temperature on oatmeal agar under cool white fluorescent lights (six each, Philips, F40T12/CW Plus, 40-W bulbs, suspended 60 cm above the plates), (2) gall-isolated fungus grown on fresh-cut autoclaved goldenrod stems placed on the surface of water agar plates, (3) egg-conidia isolates grown on oatmeal agar, (4) egg-conidia isolates grown on fresh-cut autoclaved goldenrod stems on water agar, (5) conidia collected from pycnidia found on field-collected *S. altissima* stems, or (6) conidia found in the mycangia of malaise-trapped adult *A. carbonifera* females from the HS site (Table 1). The gall and egg-conidia isolates (1–4 above) were all grown at the same time under the same conditions in a randomized complete block design. Two linear ANCOVA (analysis of covariance) models with 'width' as a covariate were used to test for significant differences in conidia length (R, version 2.8.1). The standardized residuals from these models were roughly normal, distributed mostly between the mean ± 2 SD, and appeared randomly associated with the fitted values. The first model included three explanatory variables: (a) fungal isolate source (i.e., egg-conidia isolate or gall-isolate), (b) growth media (i.e., fresh-cut *S. altissima* stems on water agar or oatmeal agar only), and (c) width as a covariate; plus (d) all the two- and three-way interactions. This model included only the first four treatments (1–4 above). The second model included all seven conidia sources with only two explanatory variables (i.e., width as a covariate, conidia source, and their interaction).

Gall initiation in field plots

Two experiments were conducted in an attempt to initiate galls on *S. altissima* accessions grown up in the greenhouse and transplanted to a common garden (WSU-1; Table 1). *Solidago altissima* rhizomes were collected from three sites [BCWMA, KWP, and Varner Road (VS) sites; Table 1] in early April and 10 clones of 10 source plants were started in a greenhouse and later transplanted to the common garden. Large conical tomato cages were placed over half of the plants in the field plot (n = 50, five replications of each accession). The cages were covered with fine-mesh sleeves (194 holes cm^{-2}) and buried about 10 cm deep. Mature galls were collected from *S. altissima* from various field sites on 9 July 2008, separated by morphotype, and placed on the ground inside each of 40 cages (10 plants were controls) in the same morphotype proportion as the total collected. Each cage received 44 irregular, seven crescent, five cushion, and four flat galls. Recently cut dried goldenrod stems, old goldenrod stems (previous year's growth), and extraneous ground litter was cut into 10-cm sections and placed in the bottom of each of the cages. It was thought that this debris might provide a source of fungal conidia for emerging adult females to collect. The caged plants were checked periodically for the formation of new galls

and the presence of emerging adults. After 24 days all plants were cut to 30 cm and checked thoroughly for the presence of galls.

In a second experiment, a subset (n = 20) of the same caged plants were allowed to re-grow for 17 days and then mature galls were added to the cages as above (23 irregular, 16 cushion, 11 crescent, and six flat galled leaves per cage) on 19 August 2008, but covered with freshly cut hay. To each of these cages was added a single *S. altissima* stem obviously infected with a pycnidia-forming fungus (presumably *Botryosphaeria* spec.) collected from the BCWMA site. Each infected stem was placed in a bottle of cut-plant solution, which was not allowed to go dry. The plants were monitored periodically for the formation of galls and after 29 days all the plants were cut to 30 cm and the cut stems checked thoroughly for galls.

Gall initiation in screen tents

Two experiments were conducted in an attempt to initiate galls on *S. altissima* accessions grown up in the greenhouse and moved to outdoor screen tents (WSU-2 site; Table 1). Thirty goldenrod accessions (presumably different genotypes), 14 of which were the same as those in the field plot experiments, were divided equally and placed in each of two 1.8 × 1.8 × 1.8-m screen tents with the floor covered with black plastic (n = 72 potted plants per tent). The tents were set up adjacent to one another and the plants allowed to stand for 8 days to ensure no galls were initiated during transport from the greenhouse to the screen tents. One tent (treated) had the floor covered with old goldenrod stems (previous year's growth) collected from a field site. The other tent was a negative control. To provide gall-initiating adults, each tent was randomly supplied with 48 *S. altissima* stems infested with a mixture of mature galls in a 20-l bucket filled with cut-plant solution on 2 July 2008. During collection of these stems, any pupal exuvia found lodged in the galls were removed. The plants were checked periodically for galls and finally after 23 days the plants were cut to 30 cm and the stems and leaves checked thoroughly for galls. During these periodic checks many adult midges were seen alighted on the screening in both tents. The total number of exuvia on the initiating cut-stem galls was tallied to reveal that at least 111 irregular, zero cushion, zero crescent, and one flat adult had emerged in the treated tent. In the control tent at least, 107 irregular, four cushion, two crescent, and zero flat adults emerged.

In a second experiment started on 13 August 2008, the same set of plants and tents were used as above, but different fungal sources were added to each tent. To one tent were added three 40-l plastic tubs (15 cm deep) filled with a plant-fungus-topsoil mixture. Holes were drilled in the plastic covers and they were elevated 15 cm above the tubs to provide shade, but allow rain water and female access to the soil surface. The soil in each of the tubs contained a mixture of 70 fresh *S. altissima* stems cut to about 5 cm long, the agar from 30 oatmeal-agar plates (100 × 15 mm) of gall-isolated cultured fungus, three handfuls of triple-ground mulch, and about 10 l of old dried goldenrod leaves. This mixture was allowed to stand for 2 weeks before the experiment started. The second tent was supplied with 54 cut *S. altissima* stems infested with pycnidia-producing fungus (presumably *Botryosphaeria* spec.) and each tent was also supplied with 100 gall-infested *S. altissima* cut stems. All cut stems were kept in 20-l buckets of cut-plant solution. Periodically and after 48 days the plants were checked thoroughly for galls. The total number of exuvia found on the cut stems was tallied to reveal that at least 29 cushion, four crescent, five flat, and nine irregular adults had emerged in the plastic-tub treated tent, and 13 cushion, four crescent, four flat, and one irregular adult had emerged in the fungus-infested-cut-stem tent.

Results

Biology and life history

The eggs of *A. carbonifera* are laid on the underside of the leaf in the vicinity of the meristem (Figure 1B). Females have up to 300 eggs at the time of emergence and fecundity appears to differ by morphotype (JJ Heath, unpubl.). The larvae (Figure 1C) hatch and the fungal spores germinate (Figure 2) within a few days of oviposition and begin to burrow/grow into the leaf tissue within a few millimetres of the ovipositional site. Once the larva has penetrated the leaf tissue, fungal growth becomes evident on the bottom and then the top of the leaf. The mean time for fungus to appear on the top of the leaf was 6.7, 8.0, and 8.2 days for cushions, flats, and irregulars, respectively. The linear model with leaf age the eggs were found on as a covariate provided estimates of the time for fungus to appear on the top of the leaf, assuming oviposition occurred on zero-aged leaves. This linear model indicated that there was a significant interaction between the age of the leaf oviposited on and the time for fungus to appear on the top of the leaf as well as significant main effects of leaf age and morphotype. The intercepts from this model were 8.6, 8.0, and 7.6 days for cushions, flats, and irregulars, respectively.

Adults (Figure 1A) emerge from the galls approximately 3 weeks after fungal growth is evident on the top of the leaf. The entire life cycle from egg to adult is about 4–5 weeks. Galls are unisexual with only rare instances of galls with mixed sexes; this is consistent with the findings reported by Weis et al. (1983) for this species and for cecidomyiids in general. In Ohio, larvae may begin to enter

Figure 1 *Asteromyia carbonifera* (A) adult, (B) eggs, and (C) larvae within a dissected gall. Adults and mature larvae are about 1–2 mm in length. Scale bar on (B) is 200 µm and eggs are typically 180–240 µm long. See online colour version.

diapause as early as the 1st week of September, but galls continue to be initiated as late as the 1st week of October. *Solidago rugosa* galls marked in the field (SC-1 site) in July and collected in December the same year still had late instars within them, indicating a very early initiation of diapause in some morphotypes. However, the physiology of these larvae may have been altered by undetected parasitoids. Larvae pupate in the spring and new galls can be seen forming in late May, but larger populations are not realized until mid to late June in Ohio. The timing and details of certain aspects of their biology may vary with gall morphotype. For instance, controlled experiments indicate that crescents can oviposit and develop on mature tissue (JJ Heath, unpubl.).

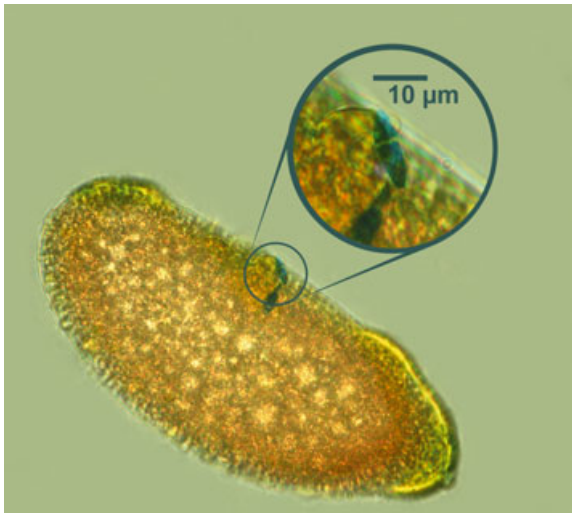

Figure 2 Two germinating fungal conidia on an *Asteromyia carbonifera* egg collected from the new growth of a *Solidago altissima* plant. Inset shows an enlargement of the conidia. Conidia stained with lactophenol cotton blue. See online colour version.

Asteromyia-fungus interdependency

In the experimental trials designed to test whether fungal growth and gall development is dependent on the midge larvae, we found that the galls in which the larva was removed ceased to grow (Figure 3), whereas the mock removals and controls were unaffected and continued to develop normally (Figure 3). However, in those trials where the larvae were touched with forceps during the mock removals the larva often died, causing these galls to cease development (Figure 3). Dissection of the galls at the end of the experiment revealed that all the galls that failed to develop contained dead larvae, whereas developing galls had healthy larvae.

Asteromyia larvae that were dissected from galls and placed on agar plates with a portion of their gall fungus (with no host-plant material) grew more than controls with no fungal inoculation ($F_{1,20} = 5.80$, $P = 0.026$; Figure 4). In preliminary experiments, some of the larvae appeared to form gall-like structures on the agar plates (Figure 5A–C). As expected, the difference between the treatments was smaller when the larvae were larger at the beginning of the experiment (i.e., a significant interaction between start length and treatment: $F_{1,20} = 6.60$, $P = 0.018$; Figure 4). Some larvae died and became completely deteriorated making a final measurement impossible; therefore, the sample sizes decreased (larva and fungus transferred, $n = 9$; controls, $n = 15$).

Adult behaviour

Field observations of 15 *S. rugosa* midges, observed for on average 2.7 h (maximum: 4.4 h), and 31 irregular midges, observed for on average 2.3 h (max: 3.8 h), provided no evidence of mating or conidia collection for either population (Figure 6). However, it is clear that females collected fungal spores somewhere in their environment, as the my-

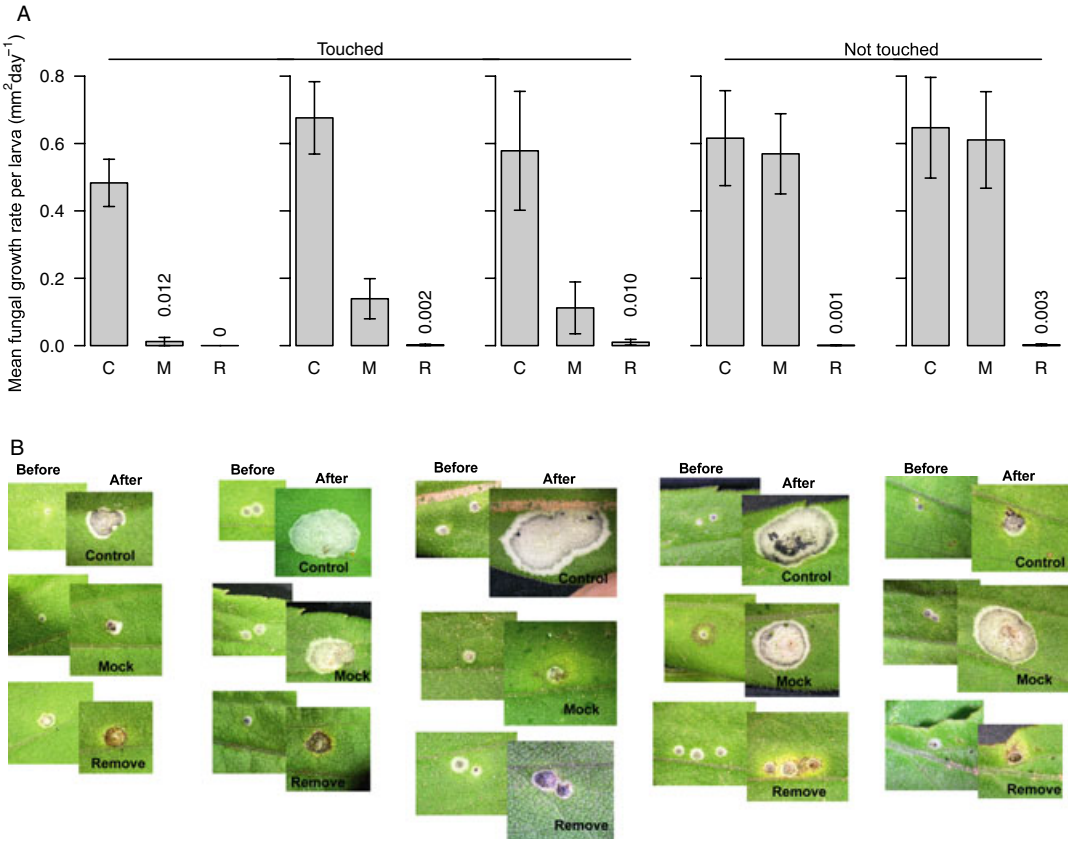

Figure 3 (A) Mean (± SEM) fungal growth rate (mm^2 per day per larva) after 10 days of incubation; organized by treatment (bars) and experiment (individual graphs). Bars are labelled with treatment abbreviations: negative control (C), mock removal (M), and complete larva removal (R). Mock larvae were either 'touched' or 'not touched' with the forceps in the indicated experiments. The values of small means are indicated above the bars. (B) Representative before and after photographs ordered by experiment. Photographs are scaled proportionately to allow comparison within and among experiments. See online colour version.

cangia of Malaise-trapped females always contain conidia (Figure 7). This suggests that these activities occur sometime after the period of our observations (Figure 6). A rare behaviour consisting of touching or dragging the ovipositor on the leaf surface was observed in two irregular females, which may be associated with conidia collection (Figure 6B). The leaves were inspected where this behaviour occurred and no eggs were found. Males and females began eclosing in the early morning as the last of the night's dew was dried from the leaves (range of eclosion times: irregulars = 06:05–08:25 hours Eastern Daylight Savings Time (EDST), rugosa = 07:39–09:00 hours EDST). Males and females of both morphotypes spent approximately 2.5 h on the bottom of the leaf from which they eclosed before flying off (mean ± SEM, males: 2.31 ± 0.34 h, n = 6; females: 2.47 ± 0.18 h, n = 22). The marked galls of both morphotypes were always checked for missed eclosions before leaving the field site for the day. In only a few cases were new exuvia or emergence holes found the following day, indicating that the majority of adults eclosed during the early morning hours. At most 5% of the marked galls had eclosions on any given day, but this was more generally 0–1%. The series of behaviours we describe (Figure 6) are nearly identical to those provided in the photographs of Gagné (1989); Plate 2, C–F) for a different gall midge species.

Fungal acquisition and gall initiation

The examination of conidia morphology from different sources indicated a high degree of phenotypic plasticity in the shape of *B. dothidea* fungal conidia. All sources of conidia (e.g., gall, stem, agar cultures) produced conidia with a range of sizes overlapping with those found on *A. carbonifera* eggs or in their mycangia (Figures 2, 7 and 8), but there were differences in shape between conidia obtained from cultured fungus and those obtained directly from midge eggs or adults. In a full ANCOVA model with all two- and three-way interactions (first model), the only

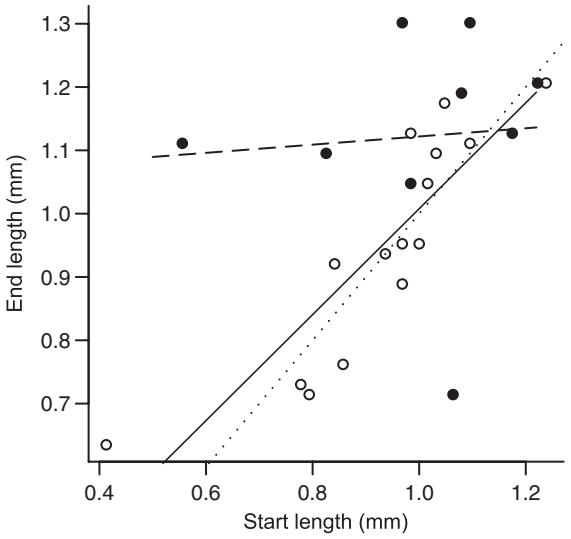

Figure 4 *Asteromyia carbonifera* larval length (mm) after 2 weeks of growth on malt-extract agar plates treated with Nystatin and with (closed circles, dashed line) or without (open circles, solid line) gall fungus added. The fine dotted line has a slope of 1 and an intercept of 0 and denotes no change in length.

significant term was the growth media (i.e., goldenrod stems or oatmeal agar), with conidia being slightly shorter when grown saprophytically on goldenrod stems (Figure 8H, top four lines; $F_{1,403} = 14.0$, $P<0.001$). Tests for positional effects were not significant. With all seven conidia sources included in the analysis (second model), width covaried with length ($F_{1,552} = 155.4$, $P<0.001$) and the effect of conidia source was highly significant ($F_{6,552} = 190.4$, $P<0.001$). There was no significant interaction of conidia width and source, indicating that the slopes were homogeneous (Figure 8). A priori orthogonal decomposition comparing wild conidia sources (Figure 8H, bottom three lines) to cultured sources (Figure 8H, top four lines) revealed that wild conidia were significantly shorter than cultured conidia ($F_{1,556} = 1\ 021$, $P<0.001$). Further decomposition of only the wild-sourced conidia concluded that goldenrod-stem conidia were significantly longer than those from the midge (i.e., eggs or female mycangia) sources ($F_{1,145} = 23.5$, $P<0.001$). Additional decomposition showed that mycangia conidia obtained from adults at the HS site were also slightly shorter than egg conidia obtained from the BCWMA and KWP sites ($F_{1,56} = 11.9$, $P = 0.001$).

Each of four experiments designed to induce the production of galls on the leaves of *S. altissima* hosts failed; not a single gall was initiated. However, galls induced by natural populations of *A. carbonifera* rapidly appeared in high numbers on a replicated set of 10 *S. altissima* accessions in an un-caged field plot immediately adjacent to the caged field plot (i.e., 435 irregular, 335 crescent, 91 cushion, and 42 flat morphotypes).

Discussion

Time required for appearance of fungus

The experiment to determine the time for fungus to appear on the top of the leaf took into account the fact that in most cases the oviposition event was not actually observed. This was attempted by incorporating the age of the leaf the clutch was found on as a covariate. If the eggs were initially laid on zero-aged leaves, then the time for fungus to appear on the top of the leaf for a given morph should be the intercept regardless of the age of the leaf the eggs were found on. This linear model indicated that there was a significant interaction between the age of the leaf oviposited on and the time for fungus to appear on the top of the leaf as well as significant main effects of leaf age and morphotype. However, the intercepts and means of this AN-

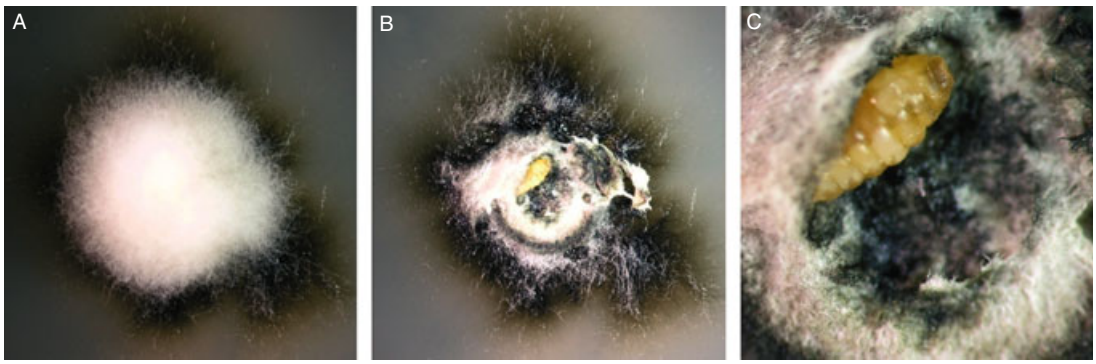

Figure 5 *Asteromyia carbonifera* pseudo-gall developing on (A) malt-extract agar. (B) Same as in (A), but it has been dissected. (C) The same as (B), but at a higher magnification to show the healthy pre-pupa inside. See online colour version.

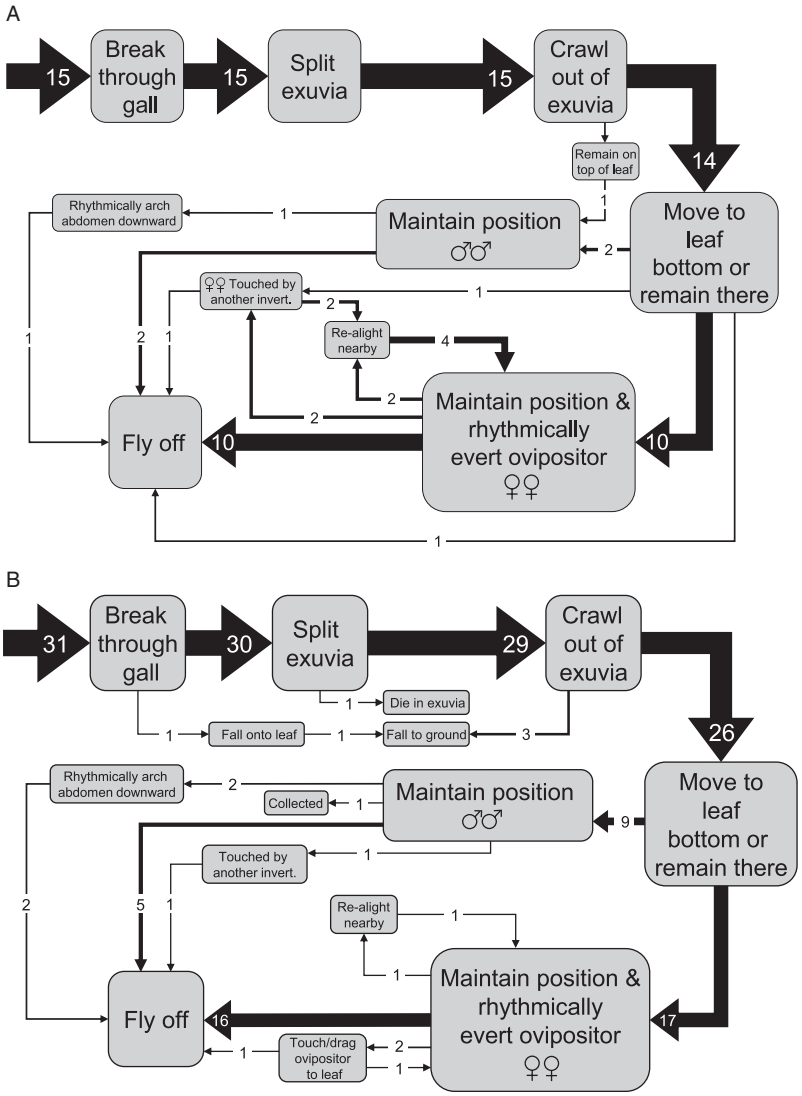

Figure 6 Ethograms of *Asteromyia carbonifera* behaviour under natural field conditions. Galls marked and observed for (A) *Solidago rugosa* morphotypes and (B) *Solidago altissima* irregular morphotypes. Behaviour was observed from eclosion to fly-off. The thickness of the arrows is proportional to the relative frequency of the particular transition from one behaviour to the next. Numbers along arrows are the absolute number of transitions observed.

COVA model were only marginally different. Furthermore, one irregular female and one flat female were actually observed ovipositing on different aged leaves (i.e., ages 0 and 3); therefore, our assumption that eggs were always initially oviposited on zero-aged leaves did not hold. These observations make the interpretation of the biological significance of this minor statistical interaction difficult. Nevertheless, with all the morphs pooled, the overall mean time for fungus to appear on the top of the leaf was 7.4 days, which is a good representation of all three morphotypes with an accuracy of ±1 day.

The nature of the *Asteromyia-Botryosphaeria* symbiosis

The results of these studies indicate that this insect-fungal system is closely knit, with strong interdependence of the fungus and the midge. This association appears to be obligate for the gall midge and with respect to the successful production of galls on *S. altissima*. The midge depends on the fungus for food (Janson et al., 2009) and protection from parasitoids (Weis, 1982b), and the fungus benefits from dispersal and requires the midge for hyphal proliferation within the context of the gall. Although the fungus likely persists independently in environments outside the

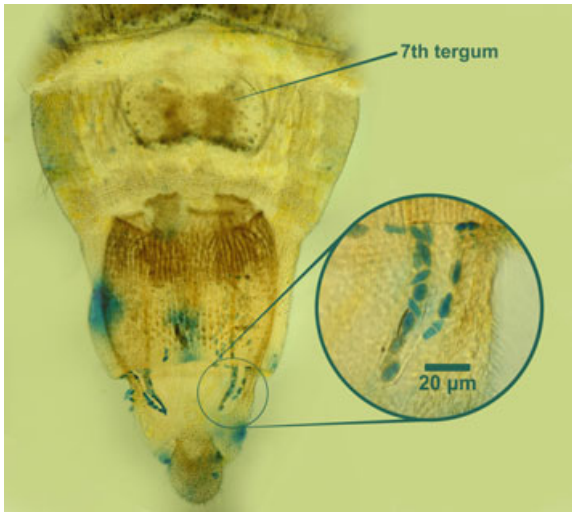

Figure 7 Dorsal view of a malaise-trap-captured female *Asteromyia carbonifera* showing mycangia filled with fungal conidia. Conidia were stained with lactophenol cotton blue. Inset is an enlargement of one of the mycangia (scale bar = 20 µm). Normally, a large proportion of the conidia would be distributed toward the anterior as well, but pressure from the slide mount forces the conidia toward the posterior in what appears to be tubes or specialized folds of tissue that lead the conidia to their point of deposition on the passing egg. See online colour version.

context of the midge galls and could have additional modes of dispersal, within the context of these galls, midge dispersal is necessary. As the gall fungus only rarely produces sporulating pycnidia, the benefits (or the costs, for that matter) to the fungus of associating with the midges remain unclear. It is possible that the midge is simply parasitizing the fungus with no reciprocal benefit. However, teasing out the costs and benefits of apparently mutualistic associations can be tricky (e.g., Herre & West, 1997); the true nature of this interaction will become clearer with a better understanding of the life histories of both players. This system is not unlike other insect-fungal associations involving Ambrosia beetles, fungus-gardening ants, and other flies. Recent work on other galling insects including cecidomyiids has found that they actively manipulate plant defensive chemistry (Tooker & De Moraes, 2007, 2008; Tooker et al., 2008). This manipulation may be a factor in allowing the fungus to proliferate and form the protective gall-like structure.

Although several larvae died when transferred from galls to agar plates, this experiment still revealed the ability of larvae to grow on a fungus-only diet, strongly supporting the hypothesis that the fungus is the larva's primary food source within the gall. Even in young galls, we have observed that mycelium quickly envelops the larva making direct access to the plant tissue difficult. Furthermore, sterol analysis of fungi, plants, and midge larvae indicates that the midge larvae obtain their sterols mainly from the fungus (Janson et al., 2009). The observation that fungal structures resembling galls formed on the agar plates, suggests that the larva is primarily responsible for the general gall structure. Feeding damage and/or salivary-gland secretions are likely responsible for the formation of the hard, black, carbonaceous material (stroma) that formed on agar plates in areas of larval grazing. This material was similar to what surrounds and protects the developing larva from desiccation and parasitism in a natural gall. Larew et al. (1987) describe a cecidomyiid from a lineage evolutionarily basal to *Asteromyia* (Bissett & Borkent, 1988) that feeds only on fungus and forms similar galls in the absence of a host plant, suggesting that the ability to form fungal galls is a plesiomorphic trait. Haridass (1987) was able to rear *Neolasioptera cephalandrae* Mani larvae to adults, solely on gall-isolated fungus growing on Petri dishes, although they observed high mortality as well.

It is clear from our consistent observations of specific conidia associated with female ovipositors and eggs that *A. carbonifera* females intentionally obtain fungal conidia, store them in mycangia, transport them, and deposit them on their eggs. The fungus in these *Asteromyia* galls has been identified as *B. dothidea* (Bissett & Borkent, 1988; Janson et al., in press), a member of the family Botryosphaeriaceae. Other fungi assigned to this genus and even species are primary and secondary plant pathogens on many important ornamental and horticultural crops. For example, grapes, avocados, oaks, apples, pears, olives, *Prunus* species, poplars, pines, ashes, elms, and various berries are known to harbour or present disease symptoms associated with *Botryosphaeria* spp. (Bonfiglioli & McGregor, 2006). *Botryosphaeria ribis* is thought to form cankers on the stems of goldenrods (Horst, 2008). Many studies have provided evidence that ascospores of this family are primarily wind dispersed, whereas conidia are primarily water dispersed (Sutton, 1981; Pusey, 1989; Ko & Sun, 1995; Ahimera et al., 2004). To our knowledge, only two studies have provided evidence for this active transportation of fungi by gall midges (Borkent & Bissett, 1985; Adair et al., 2009). No studies have investigated the direct role that cecidomyiids may play in vectoring *Botryosphaeria* spp. to other plants.

Gall initiation, conidia collection, and eclosion behaviour

Gall initiation. Although we were unsuccessful at initiating galls under semi-artificial conditions, we did discover pycnidia-producing fungus on the stems of *S. altissima*. However, two experiments including exposure of midges to these infected stems did not result in the initiation of

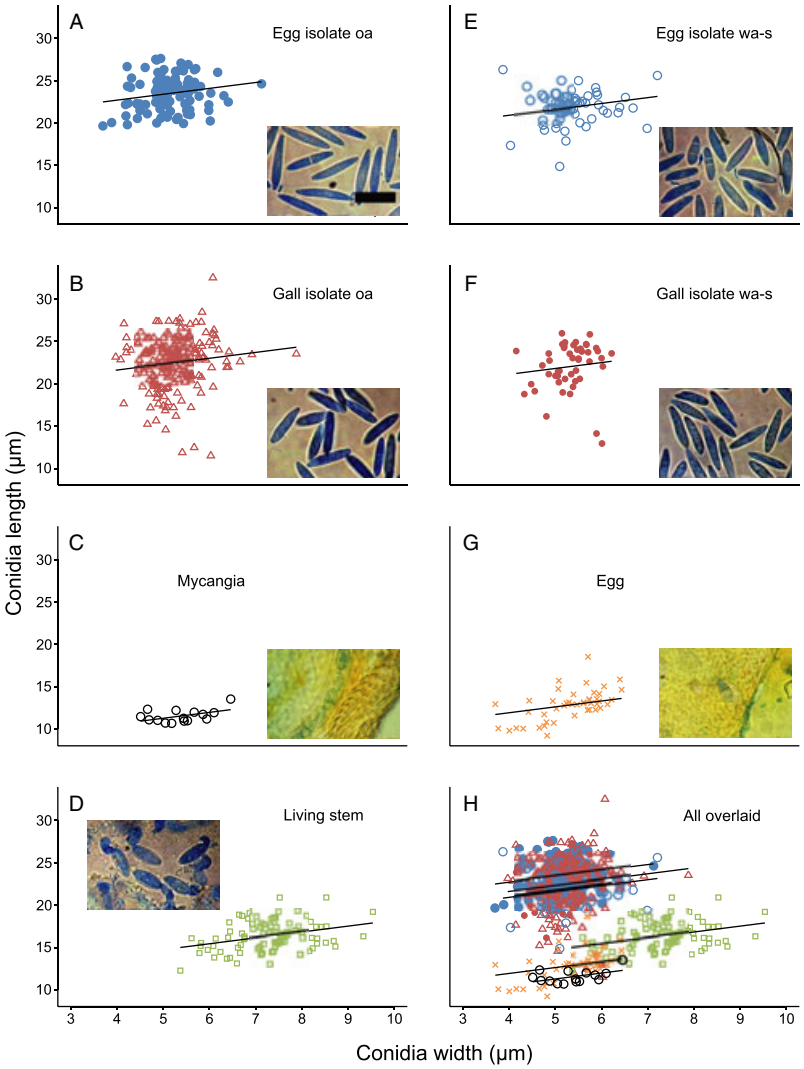

Figure 8 Length and width (µm) of individual conidia sampled from different sources. The top four panels show conidia obtained from either (B, F) gall or (A, E) egg-conidia fungal isolates, grown on either (A, B) oatmeal agar (oa) or (E, F) fresh-cut autoclaved *Solidago altissima* stems atop water agar plates (wa-s). These four sources were grown under the same conditions at the same time. The other sources are (C) *Asteromyia carbonifera* female mycangia, (D) pycnidia on field-collected *S. altissima* stems, and (G) field-collected *A. carbonifera* eggs. (H) The final panel is a composite overlay of all the graphs to facilitate comparisons. The insets are representative photographs of some of the conidia from each source. The scale bar (20 × 5 µm) in (A) applies to all the insets. Conidia stained with lactophenol cotton blue. See online colour version.

galls, suggesting that these stems are not their conidia source.

It is possible that midge behaviour was constrained in the enclosures. Many insects and vertebrates have behaviours that are fixed action patterns (Matthews & Matthews, 1978). A fixed action pattern is a sequence of stereotypical behaviours that is relatively indivisible. This indivisibility can be so strong as to prevent subsequent behaviours until the sequence is completed. Furthermore, a number of these fixed action patterns can be under hierarchical control, so that the occurrence of one may be required for initiation of another (Matthews & Matthews, 1978). It is possible that our field cages may have prevented behaviour, such as medium range dispersal, that may be necessary before conidia collection can occur. However, the field setting and use of large cages in these experiments strongly suggests that the failure of gall initiation is most likely attributable to a lack of the appropriate source of conidia.

Eclosion behaviour and mating. Our observations indicated that females did not obtain conidia from their galls as they eclosed. Observations of several female mycangia from each of the four morphotypes after they had spent at least a day in containers containing tens of galls never revealed conidia within their mycangia (n = 35). This appears to be the case in other Ambrosia gall-forming cecidomyiids (Adair et al., 2009). Our field observations also failed to reveal any evidence of behaviour associated with conidia collection during or after eclosion. Furthermore, the exuvia effectively shields the ovipositor of the eclosing female, making conidia collection from within the gall unlikely. Perhaps most importantly, no pycnidia were ever observed within or on the outside of mature galls during eclosion.

Many cecidomyiid species have sexually dimorphic antennae with the males being more plumose (Gagné, 1989). In some of these species sex-specific pheromones have been identified (Heath et al., 2005). These species tend to mate almost immediately after eclosion (McKay & Hatchett, 1984; Pivnick & Labbe, 1992; van Lenteren et al., 2002; Heath et al., 2005; Suckling et al., 2007). The antennae of *A. carbonifera* are not sexually dimorphic and attempts to attract males or females with virgin conspecifics or actively sporulating *B. dothidea* fungus have failed (JJ Heath, unpubl.). On several occasions, males and females of the same morphotype were seen emerging from galls in the field at the same time and very close to one another (i.e., within 5–30 cm), but mating was never observed. One may postulate that this is a result of inbreeding avoidance, but females have single-sex families. Therefore, these observations suggest that males and females of *A. carbonifera* may aggregate at the conidia collection site or that adults become more attractive after they have collected fungal conidia. Although *A. carbonifera* are diurnally synchronized in emergence, populations are not strongly synchronized seasonally (B Wells, pers. comm.). In our field eclosion observations, over 100 galls were marked, but less than 5% of these produced eclosing adults on any 1 day. Therefore, the probability of having a male, female, and a sporulating fungal structure present simultaneously may be quite rare. Future gall initiation studies will concentrate on one gall morphotype to increase the probability of the co-occurrence of these factors.

Conidia morphology. The morphology of conidia from different sources demonstrates that *B. dothidea* produces pleomorphic conidia and that this is affected by the growth media. The fact that egg-conidia isolates produce long slender conidia when grown as a saprophyte (i.e., on oatmeal agar or autoclaved goldenrod stems) even though the isolates originated from a population of short conidia (i.e., egg-conidia isolates), suggests that the natural source of conidia is not of a saprophytic nature. If the females obtain conidia from a saprophytic source one would expect these conidia to be long and slender, rather than the observed ovoid shape. The morphology of the conidia derived from pycnidia occurring on *S. altissima* stems in the field was more ovoid and overlapped more with those found in mycangia and on eggs, suggesting these as a possible source. However, the conidia source may be from some other plant growing in the same environment as goldenrod, such as blackberries. *Botryosphaeria dothidea* is known to attack blackberries and produce numerous pycnidia on their stems (Maas & Uecker, 1984) that overlap slightly in size with those found in the mycangia. It is also possible that the midges somehow select smaller conidia mechanically or via odours; though the benefit of this selectivity is unclear. We plan to generate isolates of the *S. altissima* stem fungus and conduct genetic analysis to determine its relationship to *B. dothidea*. Although this may be a possible source, the distribution of these pycnidia in the field is patchy and efforts to find them early in the season, when *A. carbonifera* galls were prevalent, have failed. They are much more abundant later in the season, when the goldenrod has started to form flower buds.

Although, mycangia and egg conidia were slightly different in morphology, this difference pales in comparison to the phenotypic plasticity of the fungus indicated by comparing the morphology of egg-derived conidia to the morphology of conidia derived from egg-conidia isolates in culture. Furthermore, the mycangial conidia and egg conidia came from different field sites, which may be responsible for the small but significant difference in morphology. The conidia we found in the mycangia of *A. carbonifera* females were very similar to those described by Bissett & Borkent (1988) for a variety of Ambrosia cecidomyiids that all use *Botryosphaeria* spp. to form their galls. This consistency in conidia morphology across Ambrosia gall midge taxa and a wide geographical range, suggests that conidia morphology is key to understanding where these midges obtain their conidia. However, the phenotypic plasticity associated with substrate makes it imperative that wild sources of conidia be measured. Once a source with similar morphology is found, genetic profiling and behavioural experiments will be necessary to verify its role in this system. Detailed genetic profiling might also be used to differentiate between a single fungal source and random collection by comparing the genetic variation of single-conidia isolates isolated from the same mycangia to those isolated from a single fungal reproductive structure.

We can now say that *A. carbonifera* is one of a number of complex Ambrosia gall midge mutualisms that hold

great promise to provide insight into the contribution that mutualistic relationships make to adaptive radiation and ecological speciation. Practically speaking, mating behaviour and conidia collection remain areas of future work. Without this knowledge manipulative studies to assess mating, host plant, and gall morph fidelity will be difficult and direct studies involving reproductive isolation and hybrid fitness will be nearly impossible. These issues notwithstanding, *A. carbonifera* and other Ambrosia gall midges remain tantalizing model systems for studying a range of evolutionary phenomena.

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