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Plants, gall midges, and fungi: a three-component system

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Abstract

Larvae of gall midges (Diptera: Cecidomyiidae) induce the activation of plant cells, partial cell lysis, and differentiation of nutritive tissue. Specialized nutritive tissue is essential for larval development and plays a key role in gall organization. Midges of the tribes Lasiopterini and Asphondyliini, however, do not induce nutritive tissues as part of the formation of their galls. Instead, these 'ambrosia galls' contain fungal mycelia that line the interior surface of the chambers. The fungi not only provide Lasiopterini with nutrition, they also penetrate the stems, induce the lysis of the middle lamella of host cells, and open a channel to the vascular bundles. Larvae of *Lasioptera arundinis* (Schiner) (Lasiopterini) follow the fungus and feed on its mycelium along with adjoining stem cells of *Phragmites australis* (Cav.) Trin. (Poaceae). Eggs together with fungal conidia are deposited by the imago on the host. Asphondyliini use a needle-like ovipositor to introduce fungal conidia and eggs into the organs they attack. Larvae of *Schizomyia galiorum* Kieffer (Asphondyliini) are unable to initiate the gall or to develop in the flowers of *Galium mollugo* L. (Rubiaceae) without their fungal associate. In this article, I provide an overview of oviposition behaviour in the Asphondyliini, as well as descriptions of the ovipositor and the female post-abdominal segments. Gall formation by Lasiopterini and Asphondyliini and the role of associated fungi are discussed, as is the role of the fungus as an inquiline or an organizer of gall tissues and a nutritive device.

Introduction

Among gall midges (Diptera: Cecidomyiidae), there are approximately 5 500 known gall-inducing species (Gagné, 2004) and they are remarkable in that each species induces a structurally distinct gall. Cecidomyiids have evolved various means to control the development of plant cells that comprise their galls, in order to provide their immatures with nutriment. One of the most interesting means whereby some gall midges obtain their food involves an intimate relationship with fungi (Neger, 1908; Doctors van Leeuwen, 1939; Rohfritsch, 1992b; Kehr & Kost, 1999). It has been suggested that the plant feeding habit evolved from an ancestral mycetophagous species (Mamaev, 1975; Gagné, 1989; Roskam, 1992, 2005).

Key prerequisites for moving from fungivory to phytophagy, and subsequently to gall induction, include piercing–sucking mouthparts together with the simplification of the intestinal tract and extraintestinal digestion (Mamaev, 1975). Within a few hours after the larval attack begins, wounded plant cells as well as cells of the various adjacent cell layers show evidence of cell activation (Rohfritsch & Shorthouse, 1982; Rohfritsch, 1992a). Feeding by sucking on the extended cell wall of the activated cells induces these cells to maintain a dynamic state and the galled tissue becomes a sink within the plant (Kirst & Rapp, 1974). Larval attack stimulates growth and induces tissue differentiation. Nutritive cells are essential for larval development and play a key role in gall organization (Rohfritsch & Shorthouse, 1982; Rohfritsch, 1992a).

Gall-inducing species of the tribes Asphondyliini and Lasiopterini differ from most cecidomyiids (Mamaev, 1975) in that the larval gut shows features of the ancestral mycetophagous habit, being similar to that of mycetophagous non-galling species in the tribes Cecidomyiini and Oligotrophini. In the galls of the Lasiopterini and Asphondyliini, larval-induced nutritive tissue is absent and a fungal mycelium that bears cytochemical features of typical nutritive tissue is present (Meyer, 1987; Bronner, 1992). Gall midges of the Asphondyliini attack reproductive

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structures of flowering plants as well as vegetative buds. Lasiopterini are capable of utilizing a saprophytic medium rich in fungal mycelia and the breakdown products of hard plant tissues. These gall midges damage plant stems synergistically with the associated fungus (Mamaev, 1975).

The relationship between these specialized gall midges and the fungi has been considered a true symbiosis, the fungus and the midge being mutually dependent for their survival (Neger, 1908, 1910; Doctors van Leeuwen, 1939; Bissett & Borkent, 1988; Rohfritsch, 1992b). Some work has been undertaken on the biology of these systems, especially with Lasiopterini. Eggs of Lasiopterini are deposited, together with the fungus (Rohfritsch, 1997), on the surface of target organs or in galleries/holes made by other plant-feeding insects (e.g., Coleoptera: Solinas, 1967; Coutin & Faivre-Amiot, 1981) or in vacated galls (Yukawa & Haitsuka, 1994).

There is little information on the histocytological features of the nutritive structures used by Asphondyliini, and we do not know how the imago of Asphondyliini collects, transports, and inoculates the fungus. One hypothesis is that the female scoops up fungal conidia through movement of the end of the abdomen and carries them in a large ventral pouch between the seventh and eighth abdominal segment (Bissett & Borkent, 1988; Gagné, 1989). But how the female discriminates between the different fungal species and how the right conidia are introduced into the attacked organ has never been explained. For example, Richter-Vollert (1964) observed oviposition behaviour and larval development of *Asphondylia sarothamni* Loew (Asphondyliini) on *Sarothamnus scoparius* Wim. (syn. *Cytisus scoparius*) (Fabaceae) and discovered that adult females introduced the fungus into the host plant during oviposition. It was, however, unclear as to how this occurred and how females collected and transported the fungal inoculum. Richter-Vollert (1964) suggested that the fungus is only an inquiline; a conclusion shared by Ross (1932). According to Gagné (1989), inquilines are organisms commonly found in galls that play no role in inducing the gall and do not interfere with normal gall developmental events.

According to Bissett & Borkent (1988), all gall midge-associated fungi could be assigned to *Macrophoma* [a coelomycete anamorph of *Botryosphaeria* (Bothriosphaeriaceae)]. Indeed, *Macrophoma* has been observed in *Asphondylia*-induced galls (Neger, 1910; Kehr & Kost, 1999; Veenstra-Quah et al., 2007). However, *Macrophoma* has not been found to be associated with *Lasioptera*-induced galls. *Lasioptera arundinis* (Schiner)-induced galls on *Phragmites australis* (Cav.) Trin. (Poaceae) (Skuhrava & Skuhravy, 1981; Rohfritsch, 1992b, 1997; Yukawa & Rohfritsch, 2005; Skuhrava & Skuhravy, 1992) include the fungus *Ramichloridium subulatum* (de Hoog) (a dematiaceous Hyphomycete). Galls of *Lasioptera ephedricola* Cockrell on *Ephedra trifurca* Torr. (Ephedraceae) include an ubiquitous fungus, the black yeast *Aureobasidium pullulans* (de Bary) G. Arnaud (Hermann et al., 1993).

The purpose of this study was to re-examine the conclusion that the fungus present in cecidomyiid galls is an inquiline that does not play a role in gall induction and larval development. The study systems consisted of three components, the gall-inducing insect, the fungus, and the host plant. The gall systems used for this study were *L. arundinis*, which induces galls on *P. australis*, along with the fungus *R. subulatum* and two Asphondyliini gall-inducing species, namely, *Schizomyia galiorum* Kieffer on *Galium mollugo* L. (Rubiaceae) associated with *Camarosporium macrosporium* (Berck. & Br.) (Pseudosphaeriaceae) (Meyer, 1987) and *A. sarothamni* Loew on *S. scoparius* associated with *Macrophoma* spec. (Neger, 1910). After describing the structural features of the fungal mycelia, the means by which fungal mycelia interact with plant cells will be discussed for *L. arundinis*- and *S. galiorum*-induced galls. Next, the means by which the adult females are adapted to collect, transport, and inoculate the fungal symbiont into plant tissue will be illustrated using observations of the ovipositor of *L. arundinis*, and of the post-abdominal segments of females of three Asphondyliini species: *Asphondyli websteri* Felt (host *Medicago sativa* L.), *A. sarothamni*, and *S. galiorum.*

Materials and methods

Insect material

Lateral shoots of *P. australis* (common reed) bearing galls induced by *L. arundinis* were collected in December 1994 to February of 1995 from the Parc de Pourtalés, Robertsau, Strasbourg, France. *Lasioptera arundinis* has one generation per year. Dry shoots bearing galls with mature larvae were held in a glass jar covered with muslin cloth and maintained under natural daylight conditions at 22 °C until adults emerged. Galls on *G. mollugo* induced by *S. galiorum* and *Geocrypta galii* Loew (tribe Dasineurini) were collected from Wantzenau Strasbourg, France, from June to November in 2004, 2005, and 2006. Three to four generations occur per year in both gall midge species. Because *S. galiorum* pupates in the soil, mature larvae were collected in a pot filled with a mixture of sand and garden soil. Just prior to adult emergence, the pot was placed in a cardboard shoe box connected to a small glass vessel. Adults of *A. sarothamni* were supplied by Marcela Skuhravá (Prague, Czech Republic). The imago of *A. websteri* Felt (Asphondyliini), prepared in Canada balsam, was supplied by R. Gagné (Smithsonian Institution, Washington, DC, USA). Because *A. websteri* and *A. sarothamni* have similar

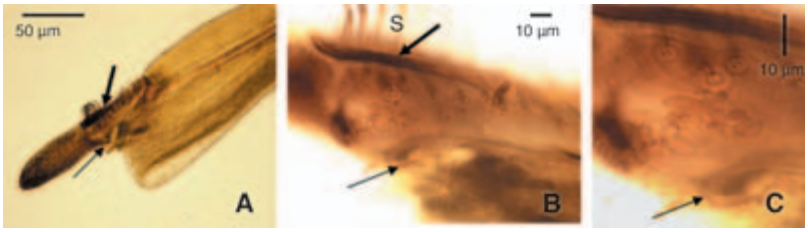

Figure 1 Ovipositor of *Lasioptera arundinis* and associated fungus. The superior sclerotized lamella (thick arrow) of uromere 10 is covered with spoon-like sensilla (s). Note the outlet of the oviduct (thin arrow). (A) Posterior part of the ovipositor with the 'mycangial' structure on uromere 10. (B) 'Mycangial' pouch on uromere 10. (C) Higher magnification of the pouch containing the conidia.

post-abdominal segments, the better preparation of *A. websteri* allowed the observation of fungal conidia along the ovipositor and the realization of good quality photos of the mycangial pouches.

Observations of fresh plant material using light microscopy

Flower buds attacked by *S. galiorum* were observed as fresh material after being treated with Gazet du Châtelier reagent (Rohfritsch, 1992c). This reagent induces partial cell maceration and renders the tissues transparent, but also stains starch, oils, cutin, and lignin. The position of the larva within the gall is easily seen, the fungus is later stained with 'trypan blue' (a benzidine-derived stain).

Histocytological techniques

The interaction of plant cells with fungal mycelia was observed using material fixed in either formaldehyde–alcohol–acetic acid (FAA) or 5% glutaraldehyde in phosphate buffer (pH 7.2). Paraffin-embedded material was sectioned at 3–5 µm and the resin-embedded (LR White; London Resin Company, EMS, Fort Washington, PA, USA) material at 0.3-µm thickness. Sections were mounted on glass slides. After paraffin removal, sections were stained with safranine-light green. The LR White-embedded materials were stained with toluidine blue O.

Behavioural observations

The fungal flora present on the leaf sheaths of the decaying stem of *P. australis* and the appendages of the ovipositor of *L. arundinis* were observed during the first hours after hatching of the imago and 1 day later. During the same time, the behaviour of the midges was observed to note where and how the midges collected the fungal conidia.

The oviposition behaviour of *A. sarothamni* has been described by Richter-Vollert (1964). The appendages of the ovipositor of *L. arundinis* as the post-abdominal segments of *A. sarothamni* and *S. galiorum* midges were observed on glass slides in Gazet du Chatelier reagent, and photographed with a Leitz microscope (http://leitzmicroscope.com).

Results and discussion

Interactions of Lasiopterini with fungi

Newly emerged females of *L. arundinis* were free of fungal conidia. A few hours (4–5) later, the midges flew around and finally landed on a distal ungalled internode of a galled, dry, and decaying stem of *P. australis*. After landing on the leaf sheath, the females moved the tip of the extended terminal segments of their abdomen (Figure 1A) back and forth over the surface of the leaf sheath, to place fungal conidia into specialized pouches. These pouches (Figure 1B,C) are paired and found near the outlet of the oviduct on the 10th abdominal segment (or uromere), beneath the superior lamella. After the female has collected conidia, she oviposits at the base of a new, fast-growing lateral shoot of a reed stem. Eggs (40–100) and fungal conidia are deposited concurrently. The ovipositor of Lasiopterini is equipped with bristles, sensory hairs, and scoop-like sensilla (Tastas-Duque & Sylven, 1989). The newly hatched larvae puncture epidermal cells of the reed stem with their mandibles; this discrete wound allows the fungus to attack the plant. Some larvae move up the stem following an opening made by the fungus (Figure 2A,B). By lysing the middle lamellae of plant cells, the hyphae induce cell dissociation and the larva moves into the space created between cells and disseminates the fungal inoculum with their unusually long spines (Figure 2D). Other larvae move through the cortical parenchyma towards the pith parenchyma. The fungus *R. subulatum* is biotrophic, feeding both intra- and intercellularly without killing cells of *P. australis*. The long and slender hyphae grow in the intercellular spaces, moving towards the vascular bundles (Figure 2D). The larva follows the fungus toward the interior of the galled stem and feeds on activated host cells, on vascular parenchyma, and on distended, protein-rich hyphae. Together with the fungus, the larvae invade the pith, and the innermost vascular tissues of the reed stem are attacked. Fungal haustoria develop in the cells of the vascular tissues and in the pith parenchyma (Figure 2E). During its final developmental

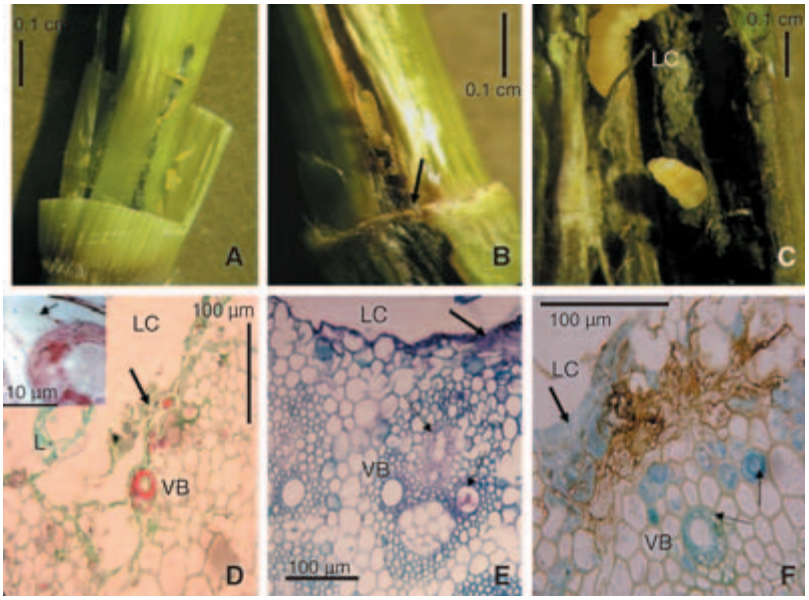

Figure 2 Lateral shoots of common reed, *Phragmites australis*, attacked by *Lasioptera arundinis* and associated fungus. The leaf sheaths have been removed. (A) Early second instars penetrate in the cortical parenchyma of the shoot, whereas first instars move stem upwards. (B) Late second instars. The fungus helps the larvae to penetrate in the pith and to migrate through the node (arrow). (C) Longitudinal section of a mature gall; larvae are in the pith within a dense mat of black mycelium. (D) Section through the cortical tissue of a young gall. The second stage larva and the fungus are in a large cavity lined by activated parenchyma cells, cytoplasma-rich fungal cells (arrow), and vascular bundles. The insert presents a first instar with long spines (arrow) on the integument. (E) Section through a maturing gall, the fungus, and the larva are in the pith: the fungus has attacked the most inward vascular bundles of the shoot (thin arrows). The third instars feed on protein-rich fungal hyphae present in the partially lysed pith parenchyma cells (thick arrow). (F) Detail of a section through a maturing gall. The partially 'digested' fungal and plant cells (thick arrow) constitute the larval food. L, larva; LC, larval cavity; VB, vascular bundles.

stages, the larva feeds on a 'near-lysed' tissue complex composed of parenchyma cells of *P. australis* and fungal, protein-rich, mycelium (Figure 2F). After the larva ceases feeding, a dense, black mycelium fills the larval chamber (Figure 2C). The relationship between *L. arundinis* and the fungus *R. subulatum* is symbiotic. Without the gall midge, the fungus cannot invade the cortical cells of *P. australis*. *Lasioptera arundinis* is adapted to collect, transport, and disseminate the fungus (Rohfritsch, 1997; Yukawa & Rohfritsch, 2005). The fungus provides three important services for the midge larvae. Without the fungus, the larva would not be able to enter the stem and access the vascular strands of *P. australis*. The fungus also allows the larva to induce cell activation, especially in the vascular parenchyma. Finally, the fungal mycelium provides the larva with a highly nutritious food.

Interactions of Asphondyliini with fungi

When female *S. galiorum* emerge from their pupal sites in the soil, they are free of conidia. How and where the female collects the conidia has never been observed. Females may find conidia in litter or by returning to old galls (Bissett & Borkent, 1988). In the tribe Asphondyliini, adult females have a characteristically large seventh abdominal sternite as well as a sharp ovipositor that allows them to pierce vegetative and floral buds or fruits (Mamaev, 1975; Gagné, 1989). Eggs and fungus are deposited by female *S. galiorum* in the flower buds of *G. mollugo*, close to the base of the anthers. Upon hatching, the larva attacks the basal portion of a stamen and also a few cells of the nearby corolla. At the same time, fungal hyphae develop from one or two germinating conidia and grow towards the floral cells attacked by the larva. The fungus develops at one or two sites near the first instar, generating a hyaline (translucent) mycelium with flat and round structures. In some places, fungal hyphae penetrate between cells and grow in the intercellular spaces. The distended hyphae, rich in cytoplasm and with thin cell walls, form a pseudo-parenchymous, translucent pad covering the wounded area (Figure 3A,B,D).

The fungus is not easily detected during the early stages of gall development, but its presence and activity are indicated by an intense accumulation of starch and anthocyanins in the apparently intact, still-closed bud,

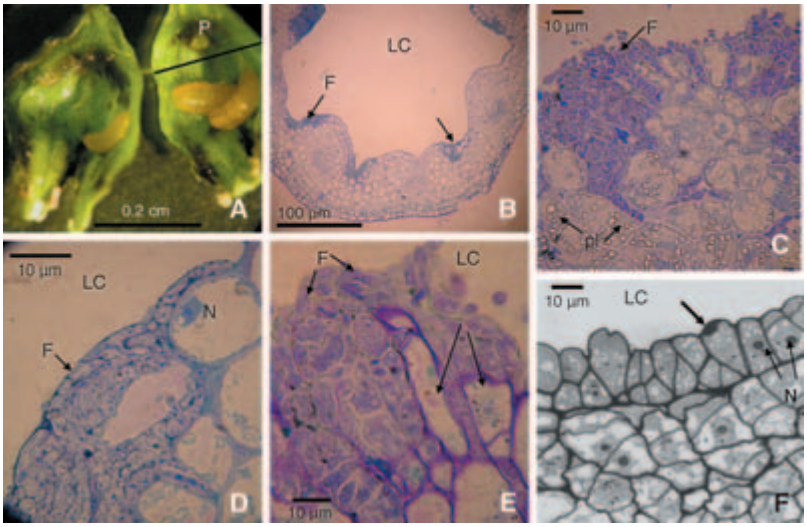

Figure 3 Sections through two gall midge-induced galls on *Galium mollugo*. (A–E) Sections through a bud attacked by three larvae of *Schizomyia galiorum* and their fungal associate. (F) Nutritive tissue of *Geocrypta galii* induced on the stem without fungal associate. (A) Longitudinal dissection of a gall used for the histocytological sections shown in Figure B–E. The line indicates the area of the tissue that has been fixed and observed; it is on this 'green parenchyma' that the larvae were feeding. (B) Transversal section of the gall with spots of fungal mycelium (arrows) between hypertrophied bud parenchyma cells. (C) Mycelium penetrating between the cells to deeper cell layers and to the vascular tissues of the attacked bud. The fungal mycelium forms a pseudo-parenchyma with protein-rich cells; it mimics nutritive tissue. Note the presence of starch in the bud parenchyma. (D) Hypertrophied epidermal cells of the flower bud in contact with the fungus. The mycelium covers the cells and penetrates in between the cells. Note the presence of a large nucleus in an attacked epidermal cell. (E) A complex of constricted plant cells (arrows) and hypertrophied, protein-rich, partially lysed fungal cells constitute the larval food. (F) Nutritive tissue induced by *G. galii* on the stem of *G. mollugo*. Small cells with a dense cytoplasm and a large nucleus provide the larva with food. The arrow shows a larval feeding puncture. P, pistil; F, fungus; LC, larval cavity; N, nucleus; pl, amyloplasts.

suggesting an important mobilization of sugars. Neither structural nor cytological modifications are evident in cells of the flower bud. The mycelium then grows along the wall of the larval chamber, becoming loosely attached to the plant cells but in some places, it encircles epidermal cells; these cells are constricted so that the fungus is able to enter intercellular spaces, reaching the cells of the second or third layer (Figure 3C) and comes close to the vascular tissues of the bud. Along the wall of the larval cavity, spots of mixed tissues, composed of modified plant cells in intimate contact with cytoplasma-rich fungal cells (Figure 3C,E), show similarities to the nutritive tissues in cecidomyiid galls without fungal association, such as the nutritive tissue induced on *G. mollugo* by *G. galii* (Figure 3F).

The fungus associated with *S. galiorum* spreads over the inner surface of the chamber of the second instar; however, in some areas, the fungus is so transparent that the larva appears to be feeding on the bud parenchyma rather than on the mycelium (Figure 3A,D). This most likely explains why larval chambers of some species are reported to be 'without a fungal mycelium', and the larva is thus assumed to feed on plant cells (Richter-Vollert, 1964; Gagné, 1989; Hermann et al., 1993). The fungus has thick, pigmented cell walls and becomes dark brown where the larva has ceased to feed (Figure 3A).

The gall structure of gall midge-induced galls of non-fungal-associated species is strictly correlated with larval development and provides information about gall midge species, larval instar, and larval fitness. In the case of fungal-associated galls, the gall structure is generally simple, no new tissues are induced, and gall size, colour, or shape are not related to larval development. Non-galled buds are green, whereas early attacked buds are reddish on the tip of the sepal and at the base of the flower (Figure 4A). The galled flower bud maintains the overall structure of the bud (Figure 4B,E,F), but often appears much enlarged (Figure 4C,D) and its colour is generally green. During the summer, small, white galls appear, which are similar to closed flowers; later in the season, large reddish galls are present (Figure 4C). Cells around the larval chamber are hypertrophied floral parenchyma cells. They have a small nucleus, large vacuoles, and starch-containing chloroplasts.

Mature larvae and fungal mycelium can be present in small galls without any new growth through cell division. Cell division often occurs at the base of the stamens, where the larva feeds. Growth by cell division and cell elongation

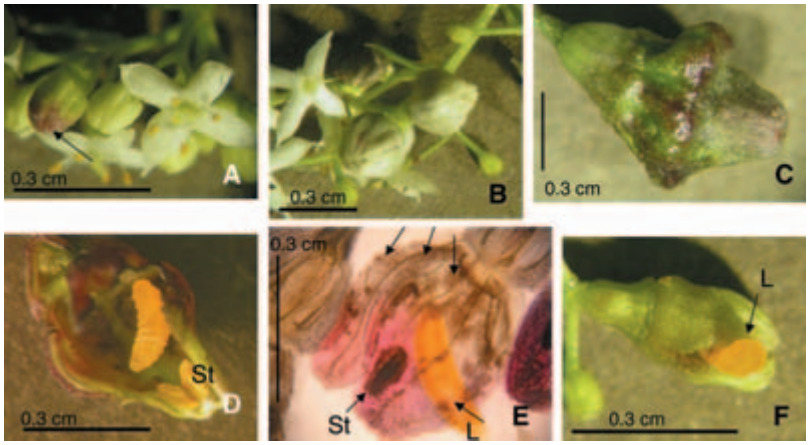

Figure 4 Different morphological structures induced by the larva of *Schizomyia galiorum* and its fungus on the flower buds of *Galium mollugo*. (A) Early attack by the first instar and its fungus: the attacked bud (arrow) is dark green and red toward its top and its base, the not-attacked bud is green. (B–F) Galls induced by mature larvae. (B) Small white galls containing a mature larva together with spots of fungal mycelium. (C) Habitus of mature gall late in the season often containing 1–3 larvae. (D) Dissection of a mature gall with a single larva. In these large galls, growth of the gall occurs between the stamen and the pistil. (E) A mature larva in a small gall where no new cell divisions have been induced. Many spots of fungal mycelium (arrows) are present along the wall of the cavity. (F) A mature larva in a small gall. St, stamen; L, larva.

causes the larval chamber to become enlarged, but the wall does not become thicker and no new tissues are formed (Figure 4D). As long as the larva feeds by actively puncturing the protein-rich cells of the fungal mycelia with its sharp mandibles (Figure 5A), the hyphae remain flat and they form a translucent pseudo-parenchyma that extends horizontally along the wall of the larval chamber (Figure 5B). Grey filamentous hyphae proliferate when the larva dies or is attacked by a chalcid parasitoid. Such galls are often larger than the galls inhabited by a healthy gall midge larva (Figure 5C) and in these galls, the hyphae grow perpendicularly to the cavity wall (Figure 5D,E). The feeding larva does not 'graze' on fungal hyphae, but punctures the thin-walled, protein-rich fungal cells. The larva controls

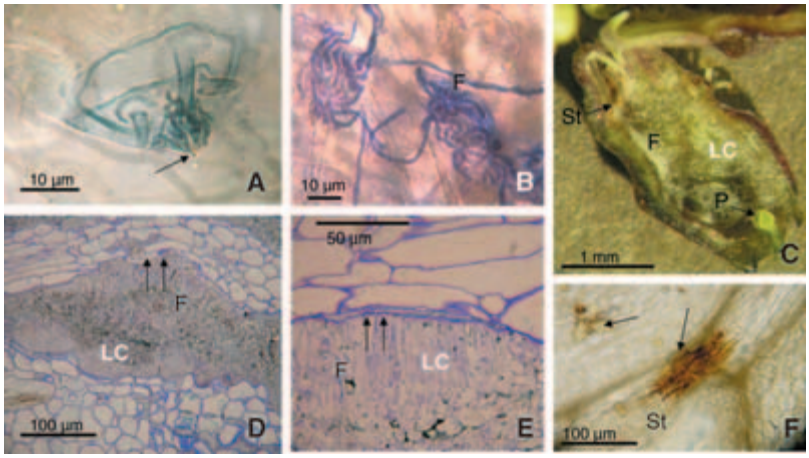

Figure 5 (A) Larva of *Schizomyia galiorum*, (B) its associated fungus, and (C–F) aborted galls. (A) Head of a second instar with sharp mandibles (arrow). (B) In the presence of a feeding larva, the fungal mycelium forms flat, protein-rich structures encircling epidermal cells. (C) Section of a large gall with a dead *S. galiorum* larva and proliferation of slender, grey hyphae. An important growth between stamen and pistil results in gall elongation. (D and E) Sections of the gall observed in (C): the grey hyphae are growing perpendicularly to the cavity wall (arrows). (F) Portion of the corolla and the base of a stamen of a bud attacked by the larva of *S. galiorum* in the absence of fungal growth. Necrotic tissues are induced at each larval feeding puncture (arrows), the gall does not develop, and the larva dies. St, stamen; P, pistil; LC, larval cavity; F, fungus.

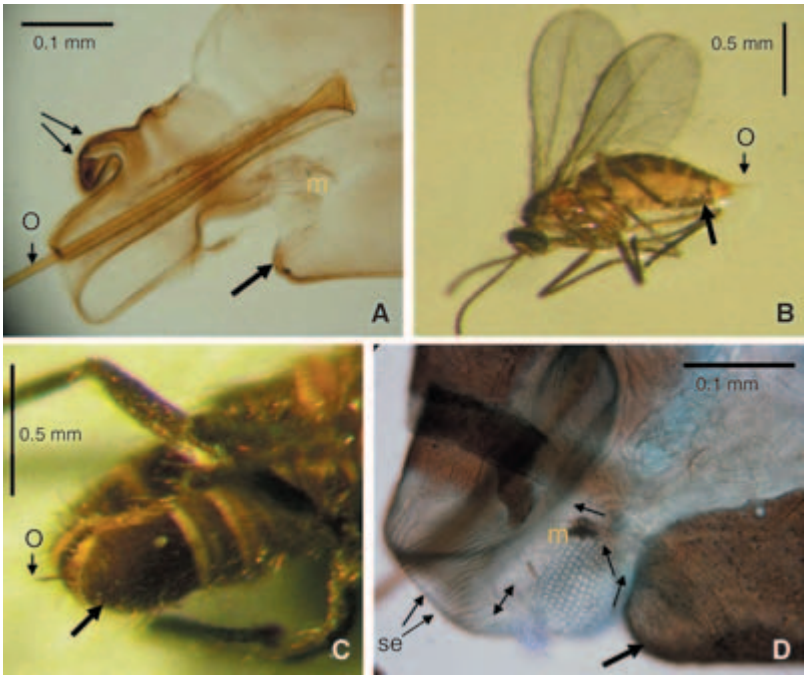

Figure 6 Female post-abdominal segments of (A) *Asphondylia websteri* and (B–D) *Schizomyia galiorum*. Note the enlarged seventh sternite (thick arrow). (A) The 'mycangial' pouch, the partially retracted ovipositor, and the dorsal lobes on the eighth abdominal segment (thin arrows). (B) Imago of *S. galiorum*. (C) Abdomen of the imago of *S. galiorum* with the ovipositor retracted and completely covered by the enlarged chitinous structure on the seventh sternite. The chitinous shield covers the 'mycangial' pouches. (D) Post-abdominal segments with the partially retracted ovipositor in segment eight and in the abdomen. The 'mycangial' pouch (m) is visible, because the chitinous shield (thick arrow) has been partially removed during the preparation. The thin arrows point to a structure that is partly chitinous and that may help in collecting and sorting the conidia. The conidia are gathered and transported in the 'mycangial' pouches. During oviposition, a groove (arrows) allows the conidia to join the setae (se) of the eighth abdominal segment. The conidia then glide along the setae to reach the ovipositor (o).

fungal growth and induces the generation of a special type of mycelium, different from the dense matt of hyphae observed by Bronner (1992) on *S. galiorum*-induced galls, but it is similar to the mycelium induced by *Asphondylia melanopus* Kieffer (Asphondyliini) on *Lotus corniculatus* Linn. (Kehr & Kost, 1999).

The fungus is essential in gall growth induction. If the fungus fails to develop, then attack by the newly hatched larva results in cell necrosis and cell wall lignification (Figure 5F). Starch does not accumulate and the development of both the gall and the larva is terminated. Thus, larval survival depends on the adult female collecting the fungus and depositing it along with the egg in flower buds. Field-collected individuals of *A. sarothamni* had a small number of conidia in their 'mycangial' pouches. The adult female of *A. websteri* (mounted in Canada balsam) (Figure 6A) showed empty pouches, but conidia could be seen between hairs on the dorsal lobes of the eighth abdominal segment and in the sheath along the ovipositor. These lobes, characteristic of all *Asphondylia* species (Gagné, 1989), are adjacent to the ovipositor and enclosed within the eighth segment on the abdomen. In addition, all Asphondyliini females have an enlarged seventh abdominal segment or sternite. This segment is particularly important for *S. galiorum* (Figure 6B–D); it bears a large ventral chitinous shield that completely encloses the retracted ovipositor. It also protects the two 'mycangial' pouches and could be used to collect conidia (Figure 6C,D). The 'mycangia' of *S. galiorum* are small (ca. 140×100 μm), but similar to those observed in *A. sarothamni*. They occur laterally, at the anterior limit of the eighth abdominal segment. The entrance of the 'mycangial' pouch is surrounded by hairs and is in contact with an additional chitinous structure (Figure 6D). Together, these structures most likely function as a 'scrub brush' useful for scratching the substratum and directing conidia towards the pouches. The pouches are linked to the ovipositor via a depression (or groove) joining the row of setae along the eighth abdominal segment.

Oviposition behaviour of Asphondyliini

To be successful, adult females must locate three different organisms: a conspecific male, a particular species of fungus, and a host plant in the correct stage for development of the larva and fungus. Because *A. sarothamni* pupates within its gall, Richter-Vollert (1964) only succeeded in observing a few adult females from emergence through oviposition. Based on the observations reported here on the 'mycangial' pockets of Asphondyliini (*A. sarothamni*, *A. websteri*, and *S. galiorum*), and Richter-Vollert's (1964) description of the egg-laying behaviour of *A. sarothamni*, the behaviour of adult females most likely proceeds in the following manner. An adult female remains stationary during the 1st day following emergence, with her head directed upwards on the shoot of the host plant. Starting at sunset, the female *A. sarothamni* becomes active, repeatedly flying upwards and then down to the soil. The females start to oviposit from the end of the 2nd day after eclosion. The female examines several buds with her long antennae and finally selects a single bud, which she visits several times. With her head oriented downwards, the midge touches the plant surface with her antennae and eventually selects the most appropriate location for oviposition. Once the oviposition site is found, the female stays firmly on the bud, extends the ovipositor, and drills it into the plant tissue at a very precise location, guided by the antennae, which are still in contact with the bud.

The ovipositor is inserted into the same location on the plant several times. After each insertion, a drop of collateral-gland secretion is deposited at the insertion site and mixes with the plant fluids that are released because of ovipositor drilling. Conidia present in the 'mycangia' are dispersed at the injured plant sites by the rapid motion of the eighth abdominal segment and the ovipositor, which remain in contact with the mixture of fluids on the bud surface. Conidia are shed from 'mycangia' onto the bud each time the ovipositor is extended. When the female retracts the ovipositor, conidia are dispersed in the oviposition channel with the secreted liquid.

The egg is then deposited into the plant tissue along with the fungal conidia; this final process takes less than 30 s. Richter-Vollert (1964) observed that only a few midges exhibited such oviposition behaviour. The majority were not attracted by the host plant and remained close to the soil, often inserting the ovipositor in the soil. This behaviour may indicate that the females are unable to initiate oviposition unless conidia have been collected.

Finally, the conclusions from these study systems can be applied to other interactions between gall midges, flowering plants, and fungi. The first instars of these fungal-associated taxa wound plant cells, and the wound enables the germination of fungal conidia and allows the fungal attack. The relationship of gall midges with fungi appears to have developed via different pathways in Lasiopterini and Asphondyliini. Gall midges of these two tribes attack different plant organs and they use different fungi and different strategies to access plant nutrients. In Lasiopterini galls, the fungus has a pectinolytic activity, with its long and slender hyphae penetrating and deeply invading plant tissues. In Asphondyliini galls, the fungus remains on the surface of the attacked organ, occasionally developing short hyphae that extend through intercellular spaces.

The two tribes have also evolved different types of ovipositor and 'mycangial' pouches. Although the transported conidia are similar in size (8–10 and 5–7 μm), they belong to anamorphs of different fungal taxa. The 'mycangia' are always minute and remain hidden behind chitinous shields. Midge larvae in the tribes Lasiopterini and Asphondyliini use the fungus to manipulate the host's defence reactions, to activate plant cells, and to extract nourishment from the host. Because the fungus has access to the vascular tissues of the host plant, it is responsible for gall growth and gall shape. The nutrients reach the larva through a structure composed of plant cells associated with protein-rich fungal cells; this cell complex mimics a 'classical' nutritive tissue as induced by gall midges without a fungal association. The physical traits (ovipositor, 'mycangia', and spines) and the behavioural traits of the imago for collecting, carrying, and inoculating specific fungal spores in its host plant indicate that the gall midge has evolved structures and behaviour as adaptations to its fungal associate. The fungus can no longer be considered as an inquiline in these galls: it is a causative agent and the gall midges must be characterized as phytomycetophages.

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