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Biology of the *Epichloë-Botanophila* Interaction: an Intriguing Association between Fungi and Insects

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ABSTRACT

*Epichloë* fungi (Ascomycota: Clavicipitaceae) are endophytes of grasses that can produce epiphytic stromata on the culms of their hosts. The fungal stromata are visited by *Botanophila* flies for feeding and egg laying. We review research over the past 20 years that has documented the heterothallic mating system of *Epichloë*, the mutualistic service of spermatization flies provide for the fungus, and host selection by flies. Flies display an active, stereotypical behavior immediately following oviposition by which spermata are transferred endozoochorously to stromata. After eggs hatch larvae feed on developing perithecia. Several studies have focused on the cost (consumption of ascospores) to the fungus of engaging in the mutualism with its insect visitors. Generally, researchers have found benefits outweigh costs suggesting the mutualism is stable, however exceptions have been reported for *Epichloë* infecting some commercially cultivated grasses. Because *Epichloë* fungi are obligate outcrossers and flies are the major (perhaps only) vector of spermata, the possibility exists that flies could promote reproductive isolation among *Epichloë* species through specific behavior. This idea has been tested using different approaches including observations of flies within screened cages containing *Epichloë* species and an analysis of fly gut content, which revealed no or very moderate selectivity by flies. Volatile fungal compounds are responsible for fly attraction and differ among *Epichloë* species. Profiles of olfactory cues that flies may use to select stromata differ among *Epichloë* species. However, in a field bioassay using electrophysiological responses by fly antennae to species-specific blends of the two predominant volatiles – a sesquiterpene alcohol (chokol K) and a methyl ester – flies showed no preference for specific blends a lack of receptor specificity. That is, flies do not appear able to distinguish between different fungal species based on their odor profiles. Thus, it appears that the flies' role in maintaining reproductive isolation among *Epichloë* species may be likely minor and that mechanisms of post-zygotic
reproductive isolation are more important in keeping compatible species distinct. However, ethological mechanisms such as 'stroma constancy' favoring intraspecific mating may operate at a local scale and need to be investigated further. Differing selection pressures on each of the interacting partners may have prevented the evolution of species-specificity in this system.

*Keywords:*
Fungus-insect interaction
Life cycle
Mutualism
Parasitism
1. Introduction

Within the realm of fungus-insect interactions, the best studied relationships involve fungal dispersal (Wheeler and Blackwell 1979; Batra and Batra 1985; Blackwell et al. 1986; Wilding et al. 1989). Other sorts of biological interactions between these diverse groups are undoubtedly common, yet have received less attention. An intriguing mutualistic interaction between *Epichloë* (Ascomycota, Clavicipitaceae) fungi and *Botanophila* (Diptera, Anthomyiidae) flies is ecologically analogous to pollination. The insects not only cross fertilize the fungus, but do so through an active behavior like that seen in the highly coevolved yucca–yucca moth and fig–fig wasp pollinating predator systems. Here we review the life cycle of the *Epichloë* fungi and the role of *Botanophila* in that life cycle, focusing on the ecological and behavioral aspects of the interaction and highlighting areas where future research is needed.

2. History

*Epichloë* contains at least 10 species of fungi that live endophytically within grasses (White, 1993; Leuchtmann, 2003) (Table 1). In the late spring or summer they can produce epiphytic stromata on culms of their hosts (Fig. 1). Well over a century ago entomologists observed that anthomyiid (*Botanophila* spp.) flies visit the stromata for feeding and egg laying (Giraud, 1872; Lucas, 1909; Trägårdh, 1913). These early investigators noted that larvae hatching from eggs feed on the fungus. Thus, they considered the flies highly specialized parasites whose diets are restricted to members of this fungal genus.

The first studies of the interaction by mycologists were by the Kohlmeyers. They documented the geographic range of the interaction in the Northern hemisphere using herbarium and living specimens (Kohlmeyer, 1956; Kohlmeyer and Kohlmeyer 1960, 1974).
They also described aspects of larval feeding and development. They documented that larvae pass through three instars and construct a brood chamber on the side of the stroma from fecal material. Larvae leave chambers in the evening to feed (T. Bultman, pers. obs.) on fungal hyphae and developing perithecia (Fig. 2Aa). During the last instar larvae often burrow into the grass stem and then eventually drop to the soil where pupation occurs. The entire larval period takes about 1 month. Adults emerge the following year with timing of their emergence closely matching formation of fungal stromata. Female flies feed only on *Epichloë* stromata; the diet of male flies is unknown, but presumably includes flower nectar. The Kohlmeyers assumed, as had others (i.e., Williams 1971), that *Botanophila* larvae found on *Epichloë* stromata all belonged to one species [*Botanophila phrenione* (Séguy)]. Yet, it is now apparent that several species (at least 6) of *Botanophila* visit *Epichloë* (Collin, 1967; Ackland, 1972; Hennig, 1976) (Table 2).

3. Heterothallism

The role of *Botanophila* in the life cycle of the fungus came under closer scrutiny once it was discovered that the fungus is heterothallic. White and Bultman (1987), in hand crossing experiments with *Epichloë etymi*, showed that individuals producing one of two mating types exist within a population: the mating system of *E. etymi*, and possibly all *Epichloë*, is heterothallic. Transfer of spermatia from a fungal individual growing on one clump of grass to a different individual living on another clump of grass is necessary for cross fertilization and continuation of the life cycle. That is, stromata are self-incompatible. Only following cross-fertilization will perithecia develop (Fig. 2Bb). Ascospores are produced within perithecia through meiosis and are the infective propagules of the fungus (Bultman et al. 1995; Chung and Schardl 1997; Brem and Leuchtmann 1999). Knowing that the fungus is
outbreeding led researchers to speculate that *Botanophila* might play a role in transferring spermatia.

4. Spermatization by flies

The first evidence of insect transfer of spermatia came from a laboratory study in which female *Botanophila* were placed in terraria containing several *E. elymi* stromata (Bultman and White 1988). Flies visited stromata for feeding and oviposition. After 24 hrs flies were transferred to individual terraria containing a single, newly-egressed stroma. Each fly was allowed to visit a stroma for 2 hr and then removed. Control stromata were concurrently placed into terraria lacking flies. Investigators found all experimental stromata produced perithecia while none of the control stromata did. These experimental results demonstrated that *Botanophila* mediates spermatization of *E. elymi* and thereby initiates the perithecial phase of the fungus, but how flies actually transferred spermatia remained a mystery. Bultman and White (1988) found spermatia clinging to the legs and mouthparts of flies, yet speculated that other mechanisms of transfer might occur.

Elucidation of the primary mode of spermatia transfer by flies came from observational studies in the laboratory. Bultman et al. (1998) found that immediately following egg laying, flies stereotypically drag the tip of their abdomen across the stroma surface (Fig. 2Cc). They walk quickly up the stroma spiraling around it and once at the top then often walk straight down the full length of the stroma. During the entire behavior the tip of the abdomen drags against the fungus and the fly's proboscis is not extended (the fly is not feeding). This behavior was only and always observed after oviposition. The authors suggested that flies were defecating spermatia during the abdomen dragging behavior.

Support for this idea came from two lines of evidence. First, if flies transfer spermatia during the stereotypical post-oviposition behavior, then one would expect perithecia to
develop in a linear or spiral pattern that corresponds to the path walked by the fly. This prediction was supported by field observations: 36 of 38 stromata with fly eggs had perithecia that initially developed (white thickening of hyphae) as distinctly straight or spiral lines. Experimental evidence came when feces of the flies were suspended in distilled water and the suspension transferred to unfertilized stromata (Bultman et al. 1998). This resulted in formation of perithecia at the sites of transfer. Thus, spermatia pass through the gut of the fly intact and viable. By visiting several stromata, a fly should accumulate spermatia of both mating types in its digestive tract and thereby effect cross fertilization when defecating after egg laying (Fig. 1). These results were quite astounding since they reveal that *Botanophila* flies exhibit an active behavior whereby spermatia are transported to a stroma. In sum, *Botanophila* plays a role in the life cycle of *E. elymi* much like that played by insects pollinating angiosperms, and further, the flies act similar to pollinating predators, like yucca moths and fig wasps, which form highly coevolved relationships that involve active pollination of their host plants (Bultman 1995). This is the first and only documentation of this type of mutualism between fungi and insects. Given that the interaction appears to involve mutualistic and parasitic components, several studies have addressed the net effect of the interaction on *Epichloë*.

5. Cost of Mutualism

A question critical to understanding the possible cost of the interaction to *Epichloë* concerned the temporal release of ascospores and consumption by fly larvae. If larvae feed on perithecia after ascospores are released, then their consumption would not reduce *Epichloë* reproductive output. On the other hand, feeding before ascospore release could substantially reduce the number of infective ascospore propagules the fungus releases. The timing of ascospore release in *E. elymi* infecting the grass *Elymus virginicus* at two sites in Missouri,
USA, was documented in the field using microscope slides coated with a sticky spray (Welch and Bultman 1995). Slides were positioned 20-30mm from stromata, collected daily for about a month, and ascospores adhering to slides counted. Perithecial consumption by fly larvae on nearby stromata was also monitored at the sites. Investigators found that ascospores were not released until 88-94% of all larval consumption had occurred (Welch and Bultman 1995). Therefore, larvae should inflict a substantial cost upon *Epichloë* by reducing the reproductive output of the fungus. Quantification of that cost came from several studies conducted on *E. elymi*.

Bultman et al. (1995) found that stromata upon which *Botanophila* deposited eggs produced nearly 2x as much perithecia as those lacking eggs, even after larval feeding was taken into account. Hence, the net effect of the fly on the fungus appears to be positive. Yet the question of how the benefits to the fungus vary as fly visitation increases and what factors might stabilize the mutualism awaited subsequent study.

In a 3 yr study of the *Epichloë-Botanophila* interaction, Bultman et al. (2000) found that flies would deposit an egg on a stroma that already possessed one or more eggs if the stroma was large. As many as 7 eggs were deposited on stromata, but the stroma surface area per egg tended to remain consistent for stromata with ≥ 3 eggs, because stromata with more eggs were larger. This general trend was also reported for a study of *Botanophila/E. elymi* in southern Missouri, USA (Parker and Bultman 1991). Stromata possessing at least one egg had 20.1% to 44.5% of their surface covered with perithecia (after larval feeding) (Bultman et al. 2000). In contrast, stromata lacking any *Botanophila* eggs had just 9.4% of their surface covered with perithecia. An explanation for how stromata lacking eggs produced perithecia may be that flies landed and transferred some spermatia (which had been clinging externally to flies) but did not lay an egg. In two of the three study years egg number and amount of perithecia produced (after larval feeding) showed a positive relationship and no
relationship in the third year. Hence, cost of the mutualism to the fungus did not increase (and if anything, tended to decrease) as the intensity of the interaction increased. This was because larval feeding was only weakly associated with the number of eggs on a stroma. Lack of over-exploitation of the fungus by *Botanophila* shows the mutualism was stable during the 3 years of the study. Analysis of the causes of mortality for fly larvae pointed to mortality from unknown causes, like disease (rather than predation, parasitism, or direct competition), which increased with increasing larval density (Bultman et al. 2000). The authors suggested the fungus was not vulnerable to over-exploitation because larvae on over-exploited stromata (i.e., those possessing many eggs) often died early and thus inflicted relatively little feeding damage on the fungus. Thus, the mutualism appears stable, apparently due to extrinsic factors (disease) which limit fly survival in a density-dependent fashion. An alternative explanation is that *Epichloë* responds to heavy egg deposition by flies with an induced chemical response [some *Epichloë* spp. can produce alkaloids toxic to insects (Leuchtmann et al. 2000; Gonthier et al. 2008)]; but this hypothesis remains untested.

Given the nature of the *Epichloë*-Botanophila interaction, study of factors that might tip the balance toward antagonism could help clarify our understanding of this interaction and of mutualism in general. One such factor is the co-occurrence of more than one *Epichloë* species. This could change host selection by ovipositing flies and thus change egg dispersion patterns across stromata. Pawlitz and Bultman (2000) studied *Epichloë clarkii* and *Epichloë baconii* at site in southern England. At one site (Farm Gate) only *E. clarkii* occurred, infecting *Holcus lanatus*. At the other site (Ashurst) *E. clarkii* co-occurred with *E. baconii* (infecting *Agrostis stolonifera*). At the Farm Gate site the absence of an alternate host was associated with aggregation of *Botanophila* eggs on *E. clarkii* which led to greater larvae mortality. Impacts of the change in egg dispersion for *Epichloë* reproductive output were not
monitored, but could provide insights into how biotic factors modify the *Epichloë-Botanophila* mutualism.

6. Reproductive isolation among *Epichloë* species

*Rationale*

For sexual reproduction *Epichloë* species depend on *Botanophila* flies as vectors of gametes. Therefore, fly activity could promote reproductive isolation, if flies were specialized to one or only a few species, much like pollinating insects in highly co-evolved systems of flowering plants.

A possible case of fly-mediated isolation may be the morphospecies *E. clarkii* on *H. lanatus*, which appears to maintain genetic and morphological distinction from *E. typhina*, although it is completely interfertile experimentally with that species (Leuchtmann & Schardl, 1998; White, 1993). Even among sexually incompatible species of *Epichloë* (biological species) genetic exchange would theoretically be possible if spermatia (gametes) and their proliferating mycelia come into contact with stroma surfaces after transfer by the fly. There is no vegetative incompatibility both within and among species of *Epichloë* (Chung & Schardl, 1997b) and, following mating, heterokaryons between male and female structures of fertilizedstromata have been observed occasionally (Chung & Schardl, 1997a). Thus, specialized behavior of flies may help to keep gene pools of different *Epichloë* species distinct.

On the other hand, sexual reproduction of *Epichloë* spp. resulting in mature ascospores was observed in the absence of *Botanophila* flies (Rao & Baumann, 2004), and conversely fly larvae sometimes developed on unfertilized stromata (Rao *et al.*, 2005), suggesting that the mutual dependence of the two partners may not operate under all
circumstances. However, *Botanophila-Epichloë* associations studied by Roa and her colleagues occurred on introduced grasses of *Dactylis* and *Festuca* cultivated for seed-production in Oregon, USA and therefore represent an artificial system where natural interactions may have been disrupted. For example, the very dense grass clumps could have allowed for gamete transfer by direct contact, or an absence of the opposite mating type would force fly larvae to develop on unfertilized stromata. Also, it may be possible that the *Epichloë* strain present in Oregon was self-compatible and thus sexual reproduction would not require gamete transfer.

*Tests of fly specialization*

The hypothesis that *Botanophila* flies are specialized on *Epichloë* hosts and as such promote reproductive isolation between species has been tested in a number of experiments and with different approaches. Flies were presented a choice of stromata from two different grass-*Epichloë* associations in five different combinations within screen cages did not prefer one association over the other (Bultman & Leuchtmann, 2003), suggesting that there was no specialization. However, results from field plots based on the analysis of ascospore progeny indicated prevalence of specific matings between stromata of the same host. Similarly, genetic analysis of spermatia contained in the fly feces showed that individual flies predominantly carried spermatia of just one species (Bultman & Leuchtmann, 2003). Thus, in this common garden setting fly visitation showed some specialization.

Another approach to test for fly specificity was taken in a large scale study based on molecular phylogenetic analyses of *Botanophila* taxa and their associated hosts (Leuchtmann, 2007). This study found six distinct fly taxa in Europe and North America feeding on *Epichloë* with up to four taxa present at the same site. However, putative *Botanophila* taxa were usually associated with more than one *Epichloë* host and thus did not confirm
specificity. Only one fly taxon appeared to be exclusively associated with *E. glyceriae* in North America. Moreover, comparison of molecular phylogenies of *Botanophila* taxa and associated hosts did not provide evidence for co-evolution of fungus and fly but rather suggested that associations of *Botanophila* with *Epichloë* fungi may have arisen more than once and that flies interacted loosely with different clades of *Epichloë* over evolutionary time. On the other hand, the intimate coordination of the live cycles and the typically mutual dependence of the two partners suggest that the interaction as a whole is co-evolved. Absence of strict specificity on a large scale however, does not exclude the possibility of selectivity by flies for particular hosts at a local scale. Indeed, there is evidence for local scale selectivity. For example, at several populations of *E. festucae* in Switzerland only a single fly taxon was present among fly larvae sampled from this host (Leuchtmann, 2007).

*Role of fungal volatiles*

If *Botanophila* flies were specialized on different *Epichloë* hosts, what would be the mechanism of their recognition? Several volatile compounds produced by fungal stromata have recently been identified and their ability to elicit a response in the olfactory receptors of *Botanophila* were tested (Schiestl *et al.*, 2006; Steinebrunner *et al.*, 2008b; Steinebrunner *et al.*, 2008c) (Fig. 3). The most prominent of the active compounds, a sesquiterpene alcohol named chokol K, was found in headspace samples emitted from fungal stromata of almost any *Epichloë* association as well as in pure cultures of *Epichloë* confirming the fungal origin of the compound (Fig. 3). The fly attracting function of chokol K was demonstrated in field bioassays using traps treated with a synthetic racemate of chokol K on which *Botanophila* flies were caught (Schiestl *et al.*, 2006). A second compound that consistently triggered an electrophysiological response in the flies' antenna was an unrelated methyl ester (MME) structurally elucidated as methyl(Z)-3-methyldec-2-enoate (Steinebrunner *et al.*, 2008c).
Amount and ratio of the two active stromata-emitted volatiles vary in different *Epichloë* species. Therefore, synthetic blends of the compounds designed to mimic volatile ratios found naturally in different *Epichloë* species were tested in field bioassays for their selective attractiveness to *Botanophila* flies (Steinebrunner *et al.*, 2008c). The different fungal specific blends attracted largely the same two fly species, although four different *Botanophila* spp. were recorded at one experimental site on nearby natural stromata. This suggests that there may be little specificity in the odor communication system of the fungus-fly interaction or that additional active compounds may be present in natural 'odor morphs'. Odor-mediated specific attraction is often achieved through a unique combination of common substances.

In a more extended genus-wide survey odor profiles including three volatile compounds produced by *Epichloë* stromata were compared both within and among species (Steinebrunner *et al.*, 2008b). There was substantial variation, yet profiles did not clearly discriminate species, but were often distinctive for particular grass-*Epichloë* associations or their geographic origin. This was particularly obvious among different host races of *E. typhina* regarding presence of the methyl ester MME and the amount of chokol K. Thus, observed odor profiles may be the result of phylogenetic constraints as well as complex selective pressures imposed by *Botanophila* gametic vectors and the local environment.

Many secondary compounds produced by *Epichloë* stromata exhibit obvious antifungal properties (Koshino *et al.*, 1989; Koshino *et al.*, 1987; Yue *et al.*, 2000). Therefore, toxic effects of the insect attracting volatiles chokol K and MME have been tested on different fungi including the *Epichloë* mycoparasite *Clonostachys* sp. (Steinebrunner *et al.*, 2008a). In bioassays employing ecologically relevant concentrations of volatiles, chokol K reduced spore germination and growth of all tested fungi significantly, while MME had no inhibitory effect. It has been proposed that the primary function of chokol K and other volatile secondary compounds was that of defense against microbial pathogens and that their
role in specifically attracting insect gametic vectors evolved later (Schiestl et al., 2006), an evolutionary pathway also suggested for floral scent evolution in angiosperms.

In summary, chemical communication in the *Epichloë-Botanophila* system appears to rely on a few specific compounds, that are able to precisely direct *Botanophila* flies to fungal stromata. However, available data do not suggest specialization of *Epichloë* species to single *Botanophila* taxa, although considerable variation in odor profiles exists. The use of unique, system-specific compounds for insect attraction as opposed to distinctive combinations of common substances is well-documented in plant-pollinator interactions (Schiestl et al., 2003; Svensson et al., 2005), and may be advantageous for *Epichloë* to decrease attraction of potential fungivores. The lack of extreme specialization at the population level by *Botanophila* and *Epichloë* species, however, does not exclude the possibility of specific gamete transfer by individual flies, e.g. stroma constancy (Bultman and Leuchtmann 2003).

**Interpretation and ecological significance**

Under a scenario of mutual benefits of *Epichloë* fungi and *Botanophila* flies, specialization at the species level is a predicted outcome. *Epichloë* is assumed to benefit from flies as reliable vectors of spermatia for cross fertilization, whereas fly larvae profit from the fertilized stromata as food sources. Clearly, the selective advantage of active fertilization to the fly, of by actively fertilizing fungal stromata, is provisioning its offspring with sufficient food provided by mature stromata. The apparent lack of specialization may not only diminish the amount of available food for the fly, but could have consequences for the reproductive success of the fungus resulting in a lowered output of sexually produced ascospores.

Spermatia of any *Epichloë* species can induce the formation of perithecia provided they are of opposite mating type (at least experimentally), however, perithecia which are the result of interspecific matings remain barren and will not produce ascospores (Chung & Schardl,
1997a). It is not known whether such abortive matings occur in nature, and mechanisms may exist that prevent improper matings when mixtures of spermatia from different *Epichloë* species are deposited on a stroma by the fly. Spermatia in mixtures may differ in their competitiveness, so that only those which are conspecific with the female structure of the stroma are able to successfully fertilize a stroma.

From the *Botanophila* fly point of view, selection pressure for developing specificity may be rather relaxed. In natural environments, *Epichloë*-infected grass clumps are often widely dispersed within a habitat. Thus, highly specific flies may need to travel considerable distances for food and oviposition, and this investment may not be compensated by an increased amount of fungal resource for the offspring. Moreover, improper matings resulting in no or incomplete fertilization do not necessarily exclude successful larval development (Rao et al., 2005), although the number of larvae per stroma that survive may be reduced.

Differing selection pressures imposed on each of the interacting partners may have prevented the evolution of extreme specialization in this system. Unique long range olfactory cues are most efficient to attract a guild of taxonomically closely related *Botanophila* species that act as gamete vectors. In the interaction flies receive food, while fungi receive conspecific spermatia for cross fertilization. It is possible that oviposition cues are more specific than long range olfactory cues, or that mechanisms of post-zygotic reproductive isolation are the primary factors keeping compatible species distinct.

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Fig. 1 – Sexual life cycle of *Epichloë*. The fungus grows endophytically in meristems and leaf sheaths of host grasses, then forms external stromata on flowering tillers around developing inflorescences (choke) with spermatia; heterothallic mating is mediated by *Botanophila* flies transferring spermatia of opposite mating type, upon which perithecia containing asci develop; filamentous ascospores are ejected and mediate horizontal (contagious) transmission through infections of host florets and then seed (modified from Leuchtmann and Schardl, 1998).

Fig. 2 – Association of *Botanophila* fly with *Epichloë*. (A) Larval brood chambers from which larvae emerge to feed on developing perithecia; (B) mature stroma after fertilization; (C) fly depositing faeces containing spermatia on stroma surface.

Fig. 3 – Chokol K is one of the predominant volatiles produced by *Epichloë* stromata that can be smelled by *Botanophila* flies. The gas chromatographic signal from flame ionization detector (FID) corresponds with the response of electroantennographic detection (EAD) using living *Botanophila* (from Schiestl et al., 2006).
Sexual Cycle

infected seed

endophytic growth

ascospores

perithecium

Botanophila fly vector

fungal stroma (choke) with spermatia