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The cost of mutualism in a fly-fungus interaction

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Abstract *Botanophila* flies act as “pollinating” parasites of the ascomycetous fungus, *Epichloë elymi*. Flies transfer fungal spermatia (gametes) among fungi as they visit their hosts for oviposition. Fly larvae consume the products of cross-fertilization (ascospores). We tested whether the cost to the fungus of engaging in the obligate mutualism rises as fly visitation increases and whether mechanisms operate to prevent excessive exploitation of the fungus. Fungi and flies were monitored over 3 years. We recorded the reproductive output of fungi, the amount of feeding by fly larvae on host reproductive tissues, and the mortality of fly eggs and larvae. In two of three years, fly eggs were randomly dispersed; eggs were clumped in the remaining year. The reproductive output of fungi did not decrease with increasing egg load; rather, fungal reproductive output tended to increase as more eggs were laid on fungi. Larval feeding was only weakly associated with the number of eggs on fungi. The lack of over-exploitation of the fungus by the fly shows that the interaction was stable during the period of time we conducted our study. Our data suggest the stability was primarily due to high fly egg/larval mortality that increased as egg load increased.

Key words *Botanophila* · *Epichloë* · Mutualism · Mycophagy · Pollinating parasites

Introduction

In contrast to studies of competition and predation, the study of mutualism has seldom been conducted within a conceptual framework (Bronstein 1994). As a consequence, while the natural history of specific mutualisms is well documented, little is understood about the general nature of the interaction. Theoretical models suggest that mutualisms should only be evolutionarily stable (i.e., able to resist invasion by nonmutualist phenotypes) when mechanisms to avoid excessive exploitation of partners are operating (Axelrod and Hamilton 1981). Failure of these mechanisms would be expected to tilt the delicate mutualistic balance to one of antagonism (Herre et al. 1999). Bronstein (1994) highlights this question as one whose answer will potentially reveal broad-based generalizations about the ecology and evolution of mutualism. Pellmyr and Huth (1994) recently presented the first empirical evidence that a mechanism exists to prevent excessive exploitation of yucca by yucca moths. In this interaction, yucca moths not only pollinate yuccas, but also deposit eggs within some flowers, where larvae act as seed predators. Flowers receiving low pollen loads and high egg counts were selectively aborted, and thereby prevented excessive exploitation of the plant.

The work with the yucca-yucca moth system highlights our lack of understanding of over-exploitation in mutualisms. Is over-exploitation indeed rare and if so, is this due to specific mechanisms that prevent it? Or rather, is the yucca-yucca moth system unusual and are many mutualisms characterized by frequent over-exploitation? Furthermore, how important is over-exploitation as a selective force maintaining differential abortion in pollination mutualisms (Richter and Weis 1998)? Answers to these questions clearly require information from additional mutualistic interactions. To augment information on mutualisms and their stability or instability, we conducted a 3-year study of the interaction between an insect and a fungus that is ecologically analogous to the interaction between yuccas and yucca

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moths. Here, we determine the relationship between insect visitation and benefit to the fungus, document how benefit and cost to the fungus scale with one another and ask whether mechanisms operate to prevent excessive exploitation of the fungus by the fly.

Materials and methods

Study system

Epichloë elymi Schardl and Leuchtman [previously *Epichloë typhina* (Schardl and Leuchtman 1999)] is an ascomycetous symbiont of grasses that produces epiphytic stromata composed of a collar of mycelium that envelops culms of its host. A stroma initially produces spermatia (gamete-like spores) that are all of one mating type (White and Bultman 1987). Following transfer of spermatia to a stroma of the opposite mating type, many perithecia (spherical ascocarps) form over the surface of the spermatial stroma. The heterokaryon that results from cell fusion of a spermatium with a hyphal cell from the recipient stroma, proliferates and meiosis occurs to form ascospores in asci within the many perithecia that form over the surface of the stroma, (Bultman et al. 1995). The perithecia are ecologically analogous to fruits and the microscopic ascospores act as propagules (analogous to seeds) that infect new grass hosts, yet this phase of the fungus' life cycle is not well understood (Bacon and Hinton 1989, 1991; Welch and Bultman 1993; Chung and Schardl 1997). The upper limit to reproduction by *E. elymi* is set by the size of the stroma. All perithecia within and among fungi are similar in size (0.2–0.3 mm diameter) (and are assumed to produce similar numbers of ascospores); however, the number of perithecia (or the proportion of the stroma surface they cover) does vary among fungi. In addition to sexual reproduction via ascospores, *E. elymi* can also reproduce asexually by infecting and being transmitted through host seed (T.L. Bultman, personal observation), as is common for some grass endophytes (Clay 1990).

The fungus has long been known to interact with anthomyiid flies of the genus *Botanophila* (Giraud 1872; Lucas 1909) [also referred to as *Phorbia* (Kohlmeyer and Kohlmeyer 1974; Bultman et al. 1995), but correctly *Botanophila* (Ackland 1972; D.M. Ackland, personal communication)]. *Botanophila* spp. transfer spermatia while visiting stromata for oviposition (Bultman and White 1988). Although female flies typically lay one egg per visit, flies subsequently visiting the stroma may also lay eggs so that stromata can accumulate several eggs over 1–2 weeks. Following oviposition, eggs hatch and larvae construct brood chambers from which they periodically depart to consume perithecia and the ascospores within them. Field work has shown that the net effect of *Botanophila* on *Epichloë* is positive; feeding damage by larvae does not negate positive effects spermatia-transferring adults have on the production of perithecia (Bultman et al. 1995). The method by which flies transfer spermatia involves a specific behavior; flies defecate spermatia during a stereotypical behavior that immediately follows oviposition (Bultman et al. 1998). Furthermore, *Botanophila* appears to be the primary, if not the only, vector of *Epichloë* spermatia (Bultman et al. 1998) and flies only oviposit on *Epichloë*; the interaction is an obligate mutualism. Hence, the *Epichloë*-*Botanophila* interaction shares many similarities with the highly coevolved systems of angiosperms, like yuccas and figs, that are both pollinated and parasitized by highly specialized insects (Bultman 1995).

A difference between *Epichloë* and yuccas or figs is that the fungus commonly reproduces asexually (through infected host seed). Nonetheless, the pervasiveness of sexual reproduction through nearly all species, argues that it is a selectively advantageous trait (Barton and Charlesworth 1998) and we assume this is true for *Epichloë*. The fact that it invests substantial energy into production of stromata suggests an advantage to sexual reproduction for the fungus. We define the cost of mutualism to *Epichloë*

as the negative effect the fly has on sexual reproductive output (i.e., amount of perithecia) by the fungus.

Study area

We established two field sites in northern Missouri, United States. Both are located in Adair Co., one (Brashear) in the flood plain of the Salt River and the other (Novinger) in the flood plain of the Chariton River. Both sites were ecotones between agricultural fields (corn or soybean) and deciduous forest. Tree species included black walnut (*Juglans nigra*), swamp white oak (*Quercus bicolor*), and shagbark hickory (*Carya ovata*). Herbaceous vegetation was dominated by Virginia wildrye (*Elymus virginicus*) and (at the Brashear site only) giant ragweed (*Ambrosia trifida*).

Procedures

At both study sites, we conducted observations during May and June in 1991, 1992, and 1994. Each year sites were visited every other day to monitor the developmental condition of stromata and the presence and development of *Botanophila* sp. (an undescribed species in the *phrenione* complex) eggs and larvae, as well as the amount of feeding by larvae on stromata. We also monitored survival of *Botanophila* eggs and larvae, categorizing causes of mortality into: predation, direct interference competition (when brood chambers coalesced one larva invariably died), and unknown causes (disease, nutritional deficiencies, mycotoxins, parasitism and possibly exploitative competition). Culms of *E. virginicus* that had *E. elymi* stromata were identified with nurseryman's tags. We visually estimated the percentage of each stroma surface that was covered by perithecia and the amount of perithecia that had been consumed by individual fly larvae. We estimated the total surface area of stromata by measuring length and diameter of each and assumed their form approximated a cylinder. After feeding by fly larvae and other consumers (Bultman and Mathews 1996) had ended and during the period when ascospores were being released from perithecia (Welch and Bultman 1993), we harvested stromata and transported them to the laboratory. There we estimated the percent of surface area of each stroma that was covered by perithecia. Five mini-transects were randomly placed along the length of each stroma and the amount of perithecia present along each transect was recorded (see Bultman and Mathews 1996). We called this "realized perithecia", since this measurement represented the reproductive output realized by the fungus. Visual estimates in the field corresponded well with laboratory estimates, and were made during the course of the study to allow us to confidently interpret the dynamic events that had produced the endpoints measured using the mini-transect method. We used our estimates of stroma surface area and visual estimates of feeding damage by fly larvae to calculate the surface area of perithecia fed upon by fly larvae. We calculated surface area because measurements expressed in percent would not reveal absolute differences in reproductive output among stromata of different sizes.

Dispersion of fly eggs among stromata was compared to the Poisson distribution using the χ^2 goodness of fit test with PROC FREQ (SAS 1985). The percent of stromata with fly eggs was compared using PROC TTEST (SAS 1985), after arcsine transformation. The effect of fly egg number on realized perithecia was analyzed by one-way ANOVA using PROC GLM, type III sums of squares (SAS 1985) and by linear regression using PROC REG (SAS 1985). *A posteriori* tests were performed using Tukey-Kramer multiple comparisons in PROC GLM (SAS 1985). The effect of egg number on stroma surface area/egg was also analyzed with one-way ANOVA. Variance analyses were conducted on ranks due to departures of data from a normal distribution (Conover and Iman 1981). The relationship between the amount of perithecia consumed by larvae and the number of eggs deposited on the stroma was analyzed by stepwise multiple regression with PROC STEPWISE (SAS 1985) using egg number, stroma length and stroma diameter as independent variables. The effect of egg

number per stroma on mortality of fly eggs and larvae was analyzed by contingency table analyses using PROC FREQ (SAS 1985). Data from 1991 were omitted because of substantial effects of millipedes on *Botanophila* survival in that year (Bultman and Mathews 1996; T.L. Bultman, unpublished work); data from 1992 and 1994 were combined for mortality analysis due to small counts in some categories for individual years.

Results

The deposition of eggs by flies did not deviate from random in two of the three years of our study (Fig. 1). The number of eggs per stroma was quite consistent, varying less than 9% over the three study seasons. Frequency of egg laying was greater at the Brashear than at the Novinger site (80.2% vs. 48.4% of stromata averaged over all three study seasons had at least one egg at the Brashear and Novinger sites, respectively; $t=4.23$, $df=4$, $P<0.013$). However, stromata were also larger at the Brashear com-

pared to the Novinger site (mean \pm SE: 246.5 ± 0.9 mm² vs. 148.7 ± 8.2 mm²) and resources per egg across all stromata were similar at both sites.

Over the three study seasons the coverage on stroma surfaces of realized perithecia averaged 9.4% for stromata lacking eggs and ranged from 20.1% to 44.5% for stromata possessing at least one egg. The level of realized perithecia was greater for stromata possessing one compared to no eggs (Fig. 2). Yet, with the exception of 1992, the level of realized perithecia tended to reach an asymptote as the number of fly eggs per stroma increased; stromata possessing at least one egg were essentially insensitive to the number of eggs they possessed (Fig. 2). We truncated egg number at three or more due to the small number of stromata in those categories. This truncation did not mask a drop in reproductive output by fungi with more than three eggs – the most eggs observed per stroma was seven and even in these, the number of realized perithecia was not lower than that of

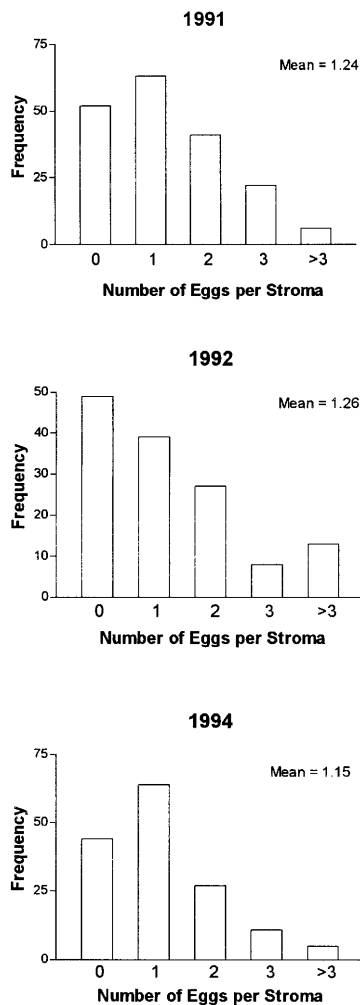


Fig. 1 Dispersion of *Botanophila* eggs among stromata. Results of comparison to Poisson distribution were as follows: eggs were distributed randomly among stromata in 1991 ($\chi^2=1.41$, $df=3$, ns) and 1994 ($\chi^2=2.8$, $df=3$, ns), and clumped in 1992 ($\chi^2=23.5$, $df=3$, $P<0.05$, index of dispersion=1.4). Data from the two study sites were combined for all figures

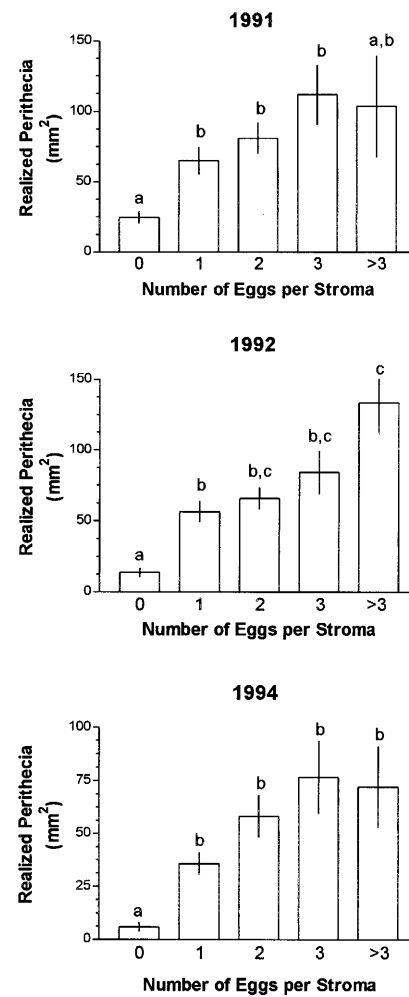


Fig. 2 Realized (following mycophagy) perithecia of *Epichloë elymi* as a function of the number of eggs per stroma (data from two study sites were combined). Error bars equal ± 1 SE. Histogram with different letters are significantly different from one another

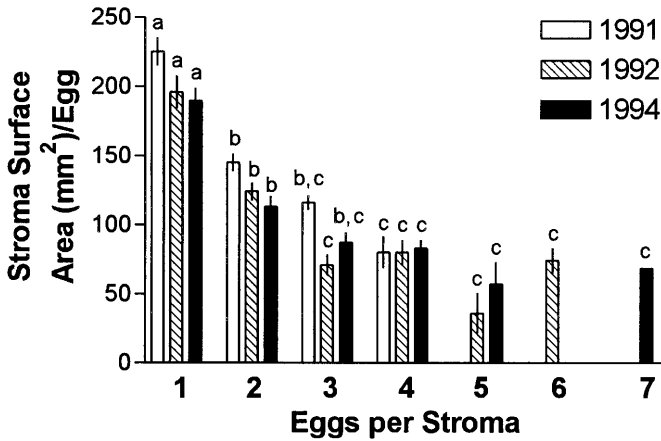


Fig. 3 Mean surface area of stromata per fly egg for each study season. Histograms within years with common letters did not differ from one another as shown by Tukey-Kramer multiple comparisons (SAS 1985)

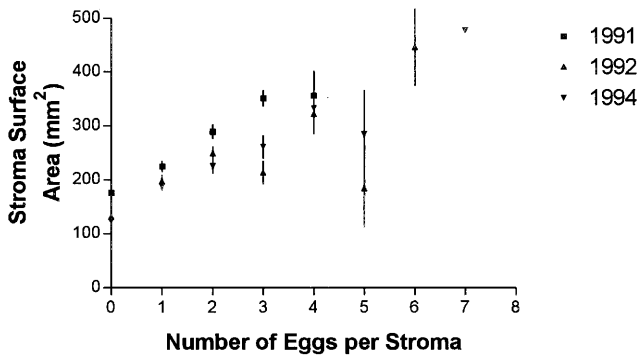


Fig. 4 Relationship between the surface area of stromata and the number of *Botanophila* eggs deposited on them; r^2 values and associated probability levels for hypothesis of nonzero slopes were: $r^2=0.86$, $P<0.02$; $r^2=0.53$, $P>0.05$; $r^2=0.91$, $P<0.001$ for 1991, 1992, and 1994, respectively

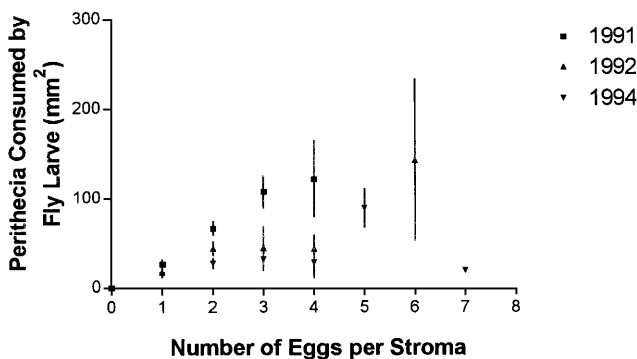


Fig. 5 Amount of *E. elymi* perithecia consumed by *Botanophila* fly larvae as a function of the number of eggs per stroma. Points represent means within each year, averaged over the two study sites. Stepwise multiple regression (SAS 1985) of surface area of perithecia consumed by larvae was regressed against egg number, stroma length, and stroma diameter. In 1991 egg number and stroma length entered the model, giving an r^2 value of 0.996 ($F_{2,4}=277.7$, $P<0.01$). In 1992 only stroma length entered the model, giving an R^2 of 0.920 ($F_{1,6}=57.6$, $P<0.001$). In 1994 none of the three independent variables were related to the amount of perithecia consumed

Table 1 Results of regression analysis of egg load vs. mean realized perithecia for each study year; t is the Student's t -statistic associated with testing the H_0 that slope=0

Year	r^2	Slope	t	df	P
1991	0.825	20.4	4.45	1	<0.05
1992	0.720	25.5	4.05	1	<0.01
1994	0.378	7.4	2.16	1	n.s.

Table 2 Mortality of *Botanophila* eggs and larvae on *Epichloë* stromata with differing numbers of fly eggs. Contingency table analysis showed overall mortality depended upon the number of eggs per stroma ($\chi^2=12.9$, $df=3$, $P<0.01$)

	Number of eggs per stroma			
	1	2	3	>3
Frequency surviving (%)	17 (16.5)	14 (13.0)	1 (1.8)	3 (3.9)
Total frequency dying (%)	86 (83.5)	94 (87.0)	56 (98.2)	73 (96.0)
Frequency dying from unknown causes (%)	63 (61.2)	71 (65.7)	46 (80.7)	55 (72.4)
Frequency dying from competitive interactions (%)	0 (0)	3 (2.8)	4 (7.0)	4 (5.3)
Frequency dying from predation (%)	23 (22.3)	20 (18.5)	6 (10.5)	14 (18.4)

stromata with fewer eggs. In fact, the opposite was true for two of the three study years; regression analysis of egg number versus realized perithecia showed a positive relationship in 1991 and 1992 and no relationship in 1994 (Table 1).

To assess the negative impact of fly larvae on the level of realized perithecia, we compared the quantity of food available to larvae and the actual amount of feeding by larvae when occurring on stromata with differing numbers of eggs. We found that as up to three eggs were added to fungi the amount of stroma surface area (food resource) per fly egg decreased (Fig. 3). This occurred even though stromata with multiple eggs tended to be larger (Fig. 4). The percentage of perithecia per stroma consumed by fly larvae over the three study seasons ranged from 8.7% for stromata with one egg to 21.7% for stromata with three or more eggs. Feeding damage by larvae tended to become more variable as more eggs were added to stromata. Stepwise multiple regression showed that in only one of the three study years did egg number enter into the model as a factor that significantly explained variation in feeding damage to fungi (Fig. 5).

We also found that the likelihood of flies surviving through the larval stages was influenced by the number of eggs deposited on stromata; overall insect mortality increased as the number of eggs occurring on a stroma increased (Table 2). Furthermore, the majority (76.1%) of mortality was attributed to unknown causes; predation and interference competition were far less common causes of death. The cause of mortality (unknown causes vs.

predation and interference competition combined) depended upon egg load ($\chi^2=8.3$, $df=3$, $P<0.05$).

Discussion

Epichloë stromata often possess multiple *Botanophila* eggs; there is no evidence for avoidance by flies of stromata already possessing eggs (Fig. 1). Variability in the intensity of the *Botanophila/Epichloë* interaction introduces the possibility that the balance between costs and benefits for both species may differ as the number of eggs deposited on a stroma changes. We specifically asked if the reproductive output of *Epichloë* varies as a function of *Botanophila* egg-laying.

The overall effect of flies on *Epichloë* reproduction is positive. In each of the three years of our study, the amount of realized perithecia was greater for stromata that possessed fly eggs compared to those that lacked eggs (Fig. 2). Furthermore, there was no evidence that greater visitation and oviposition by flies led to reduced reproduction by the fungus. Even in 1992 when eggs were aggregated on stromata (Fig. 1), realized perithecia did not decline with increasing egg number. Rather, realized reproductive output of *E. elymi* increased with egg number in two of the three study years (Table 1).

A trend for greater consumption by larvae as egg number increased occurred in only one of the three study seasons (Fig. 5). Overall, the linkage between egg number and larval consumption was not strong enough to reduce realized perithecia produced by *E. elymi*. This pattern is important because it shows the fungus was not vulnerable to over-exploitation by the fly. If higher oviposition led to levels of realized perithecia at or below that for stromata receiving no fly eggs, then the mutualism would be unstable and it would quickly change to parasitism. We do not mean to imply that the interaction is based on cooperation rather than conflict, but rather that the selfish interests of the fly (consumption of the fungal fruiting body) are not fully realized. Fly larvae, even when numerous, seldom consume all of the perithecia on a stroma.

Compared to the *Epichloë/Botanophila* system, other pollinating-parasite systems show variable effects of insect visitation on plants. Fig seed production may show positive, negative or no correlation with visitation/oviposition by fig wasps; however the commonest relationship appears to be a negative one (Bronstein 1992; Anstett et al. 1996). This variability is at least partly due to variation in inflorescence size among fig species. In contrast to figs, globe flowers (*Trollius europaeus*) appear to be insensitive to variation in visitation frequency by *Chiastocheta* flies (Pellmyr 1989), yet this interaction has received much less study than the fig system. While published data from yuccas are not available, lack of pollen is probably more important in determining fruit set than is damage from yucca moths, yet the relationship between visitation and seed production is highly variable due to variation in the amount of pollen carried and transferred by individual moths (J.

Addicott, personal communication). Nonetheless, selective flower abortion (Pellmyr and Huth 1994) may serve in the long term to limit seed loss due to feeding by moth larvae in flowers with high egg loads. In sum, within the limited data that exist, there is no clear trend for high visitation to diminish plant or fungal reproductive output. It is noteworthy that the best example of high visitation reducing seed production comes from figs (Anstett et al. 1996, 1997). Of the four mutualism systems, only figs have a short-term advantage from producing insects. This is because female wasps collect pollen from the hosts within which they developed. Hence, there may be stronger selection on figs to tolerate high visitation because there is a direct link between egg/larval load and male function of fig flowers. This direct link is absent in yucca, globe flowers, and *Epichloë*. Globe flowers and *Epichloë* must all accept insect eggs (to promote male function) but there should be no short-term selection for these hosts to cultivate the eggs/larvae – and it appears that they often do not.

The lack of excessive exploitation of *Epichloë* requires explanation, since the average amount of perithecia on a stroma a successful fly larva (i.e., survived to prepupal stage) consumed during its development was $31.6\pm 2.4\%$ (mean \pm SE for all three study seasons) of the perithecia and three or more eggs frequently accumulated on stromata (Fig. 1).

One hypothesis that may explain the insensitivity of *Epichloë* to *Botanophila* egg load is that fly larvae experience greater intraspecific competition as egg load increases. This situation would be similar to the fig, *Ficus pertusa*, in which synconia entered by more foundresses produce fewer fig wasps, apparently due to competition among ovipositing females (Bronstein 1988). If food resources become limiting for *Botanophila* larvae as egg load increases, then we would expect larval consumption to not increase linearly with egg load. Indeed, we found only a weak association between larval feeding and the number of eggs on stromata (Fig. 5). Larval damage tended to rise as eggs were added, but the amount of damage became increasingly variable as more insects shared a stroma. If intraspecific interactions become so strong that they lead to larval mortality, then one would also expect *Botanophila* mortality to be positively correlated with egg load. Again, this expectation was supported (Table 2); however, very little egg/larval mortality was due to direct interference competition (when brood chambers coalesced).

A difficulty with the competition hypothesis is explaining why selection has not modified the oviposition behavior of flies to distribute eggs more evenly among stromata to avoid the negative impact of crowding. Stromata with multiple eggs were larger than average, and hence flies did apportion eggs fairly equitably among stromata with three or more eggs (Fig. 3). Yet, one might expect flies to evolve to more completely utilize the food resource. Instead, stromata possessing fly eggs produced substantial amounts of perithecia, even after feeding by fly larvae. Again, the reason for this is that most larvae died and therefore the level of consump-

tion was far less than it could have been. This is a situation not unlike that reported by Nefdt and Compton (1996) for 11 African fig species. They found that foundresses were generally in short supply, preventing wasps from over-exploiting their hosts. While fly eggs are not in short supply, healthy larvae are. Our results suggest the best explanation for why flies do not over-exploit *Epichloë* is high levels of fly mortality due to unknown causes, perhaps disease. Additionally, there is a trend for unknown causes of mortality to increase as more eggs are deposited on stromata (Table 2). Brood chambers of larvae are produced from larval frass and are open at both ends. They should be easily colonized by bacterial and fungal spores. This could explain the high mortality of larvae on stromata possessing only one egg and may also explain the tendency for mortality due to unknown causes to increase with increased larval density. An alternative explanation for mortality is parasitism by parasitoids (Kohlmeyer and Kohlmeyer 1974). We did not observe parasitism, but at least some parasitoids of *Botanophila* spp. emerge from pupal hosts and are difficult to detect without destructive sampling.

In summary, while the *Epichloë-Botanophila* interaction appears to be a balanced antagonism, our data suggest it was not delicately balanced over three years of our study. Greater fly visitation could potentially result in excessive exploitation of the fungus, but this appears unlikely to occur. A feature responsible for the stability is greater levels of egg/larval mortality, perhaps due to microbial disease, as egg load on *Epichloë* increases.

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