Oviposition Preference of *Botanophila* Flies (Diptera: Anthomyiidae) Towards Stroma Size of *Epichloë* (Hypocreales: Clavicipitaceae) Hosts

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ABSTRACT   Stromata of grass-infecting fungi from the genus *Epichloë* (Clavicipitaceae: Ascomycota) serve as a food source and egg-laying surface for flies of genus *Botanophila* (Diptera: Anthomyiidae). Larger stromata should make it possible for flies to lay more eggs and provide more food to offspring. This hypothesis was tested in four different grass–fungus associations that occur in central Poland. In two of these associations, *Epichloë bromicola* on *Elymus repens* and *Epichloë typhina* on *Puccinellia distans*, flies showed a preference for longer stromata, and egg density on these stromata was significantly higher than in the other two associations. A negative correlation between egg density and offspring success was observed in only one association, *E. bromicola–El. repens*. However, offspring success in this association did not differ significantly from offspring success in associations with lower egg density on the stromata, in which flies showed no preference for the stroma length. Long-term observations (2000–2010) of fly–fungus interaction in the *E. typhina–P. distans* association showed that fly preference toward stroma length may vary over time but with no clear tendency. No significant correlations were found between the larval density on a stroma and either larval weight or mortality. The results of the current study question our assumptions that egg laying depends on the stroma length and the fate of eggs laid (i.e., their hatching success and the condition, in terms of weight and survival, of the larvae) on egg density. It is possible that flies choose stromata based on attributes other than size.

KEY WORDS   Anthomyiidae, *Botanophila* sp., *Epichloë* sp., oviposition preference

In nature, organisms always co-occur with other organisms from different taxa and most of these co-occurring organisms interact with each other. At present, we know that relationships between organisms present a continuum of intensity and of gains and losses that vary in time and space (Bronstein 1994a,b; 2001). Many studies show that there are places in space where a species does not enter any interaction (so called "cold spots") and places in which the relationship between partners is strongest (so called "hot spots"; Gomulkiewicz et al. 2000, Thompson and Cunningham 2002). Hence, interspecific interactions are difficult to define unambiguously.

A spectacular example of interactions that are currently studied is the relationship between *Botanophila* flies (Diptera: Anthomyiidae) and fungi from the genus *Epichloë* (Clavicipitaceae: Ascomycota). This relationship resembles interactions between some insects and flowering plants in which insects both pollinate and parasitize a plant (pollinating seed parasites). During pollination, insects lay eggs in visited

flowers and, after hatching, larvae feed on developing seeds (e.g., Pellmyr and Huth 1994, Anstett et al. 1997). Our research, which focuses on interactions between *Botanophila* flies and *Epichloë* fungi (Leuchtmann 2007; Górzyńska et al. 2010, 2011; Lembicz et al. 2013), follows this line of study.

The ancestors of those *Botanophila* flies that later became connected with *Epichloë* fungi, were likely grass herbivores initially, similar to many modern representatives of the family Anthomyiidae. Those individuals who also ate fungal fruiting bodies called stromata, which appeared on grasses at some point in time, gained an advantage over other individuals because stromata were a richer source of nutrients than grass tissues (Bultman 1995). At present, stromata of fungi are the only known food source for some *Botanophila* species (Kohlmeyer and Kohlmeyer 1974). However, the *Epichloë–Botanophila* interaction is not entirely restricted to the feeding relationship. *Botanophila* females lay eggs on *Epichloë* stromata and transfer spermatia of two different types in their faeces, thus enabling cross-fertilization of these heterothallic fungi (Bultman and White 1988; Bultman et al. 1995, 1998; Bultman and Leuchtmann 2008). Some volatile compounds produced by *Epichloë* fungi are also known to attract flies (Steinebrunner et al. 2008b).

Botanophila flies have long been considered the main, if not only, vector of fungal spermatia (Bultman

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Table 1. Number of stromata collected in particular grass–fungus association and their origin

Grass–fungus association	Site	Location	Fly taxa	No. of collected stromata							
				2000	2001	2002	2003	2006	2008	2009	2010
D. glomerata–E. typhina	Morasko	52° 27.857′ N 16° 55.868′ E	*Botanophila phrenione* (Séguy) *Botanophila dissecta* (Meigen)					55			
	Pakość 1	52° 47.531′ N 18° 06.118′ E	*B. phrenione*			30	30	55	127	29	130
P. distans–E. typhina	Janikowo	52° 46.384′ N 18° 08.032′ E	*B. phrenione*	67	30	30	30		144	145	147
	Giebnia	52° 46.544′ N 18° 06.190′ E	*B. phrenione*		30	30	30	30	146	149	147
	Pakość1	52° 47.284′ N 18° 06.257′ E	n.d.	60	30	30	30	30	143	150	
	Pakość 2	52° 47.293′ N 18° 06.721′ E	*B. phrenione* Taxon 1						149	148	146
El. repens–E. bromicola	Pakość 1	52° 47.397′ N 18° 06.064′ E	*Botanophila lobata* (Collin) Taxon 1								54
H. lanatus–E. clarkii	Morasko	52° 27.910′ N 16° 55.370′ E	*B. phrenione*								67

et al. 1998); thus, the insect–fungus interaction was recognized as obligatory. At present, we know that stromata can be fertilized without the fly's contribution (Rao and Baumann 2004; Górzyńska et al. 2010, 2011); therefore, the fungus can reproduce sexually in the absence of the insect. However, the *Botanophila* life cycle depends strictly on the fungus because the fly larvae, like the adults, feed on fungal stromata until their metamorphosis.

The nutritional dependence of *Botanophila* larvae on fungal stromata suggests that a pregnant fly will select larger stromata on which to lay eggs, to ensure that more food will be available to its offspring. Based on this assumption, the aim of this study was to evaluate 1) the relationships between stroma size and both fly presence and the number of eggs laid, 2) the effect of egg density on offspring success, and 3) the effect of larval density on larval survival and weight. The investigations were conducted in four different grass–fungus associations. One of these associations has been observed since 2000, which made it possible to determine whether this interaction has changed over time.

Materials and Methods

Collection of Material. The research was conducted from 2000 to 2010 and involved three fungal species occurring on four grass species in six localities situated in central Poland (Table 1). In each of these localities, *Epichloë* stromata were sampled from study plots of 25

m^2, which were located within fungus-infected grass populations. Stromata were collected at the end of June, after the maximum number of eggs was laid.

Measurements. The presence or absence of evidence of insect visits was determined based on the occurrence of eggs on stromata, which were observed under a microscope (Olympus SZ61-TR; Olympus, Tokyo, Japan). To determine the relationship between stroma length and egg number, stroma length was measured and the number of eggs on each stroma was determined. The number of eggs comprised both the number of unhatched eggs and larvae. In addition, density of insects on a stroma was determined as the mean number of eggs per 1 mm of stroma. To characterize the effect of egg density on the offspring success, the following parameters were determined for each stroma: 1) egg density per 1 mm^2, 2) offspring success, expressed as the percentage of hatched eggs, and 3) stroma length. The comparison of the aforementioned traits was conducted between the four grass–fungus associations for the stromata collected in 2010, within the *Puccinellia distans–Epichloë typhina* (*P.d.–E.t.*) association for the years 2000–2010, except 2004, 2005, and 2007, and between the *P.d.–E.t.* and *Dactylis glomerata–E. typhina* (*D.g.–E.t.*) associations in the locality Pakość 1 for the years 2000, 2003, 2008, and 2009. The last comparison was conducted to show whether there are any differences in the studied traits of the fly–fungus relationship, if this relationship occurs in the same

Table 2. Presence of *Botanophila* eggs on fungal stromata and the relationship between stroma length and egg presence in each grass–fungus association in 2010

Grass–fungus association	N	Egg presence n (%)	Stroma lenght (mm) ± SD		P Levene's test	t	df	P
			Eggs present	No eggs				
P.d.–E.t.	440	134 (30.4)	17.23 ± 3.85	14.85 ± 3.79	NS	6.04	438	<0.001
D.g.–E.t.	130	115 (88.5)	48.19 ± 13.37	45.20 ± 14.18	NS	0.81	128	NS
El.r.–E.b.	54	54 (100)	28.50 ± 4.91	–	–	NS	–	–
H.l.–E.c.	67	11 (16.4)	31.36 ± 5.16	28.63 ± 6.81	NS	1.26	65	NS

P.d.–E.t., *Puccinellia distans–Epichloë typhina*; *D.g.–E.t.*, *Dactylis glomerata–Epichloë typhina*; *H.l.–E.c.*, *Holcus lanatus–Epichloë clarkii*; *El.r.–E.b.*, *Elymus repens–Epichloë bromicola*.

Table 3. Correlation between stroma length and *Botanophila* egg number and between egg density and offspring success in each grass–fungus association in 2010

Grass–fungus association	N	Stroma length ± SD	Egg no. ± SD	r	P	N	Egg density ± SD	Offspring success ± SD	r	P
P.d.–E.t.	440	15.58 ± 3.96	0.34 ± 0.56	0.291	<0.001	134	0.07 ± 0.02	0.70 ± 0.44	−0.073	NS
D.g.–E.t.	130	47.84 ± 13.45	2.06 ± 1.52	0.158	NS	115	0.05 ± 0.03	0.76 ± 0.36	0.003	NS
El.r.–E.b.	54	28.50 ± 4.91	1.91 ± 0.94	0.474	<0.001	54	0.07 ± 0.03	0.87 ± 0.22	−0.412	<0.01
H.l.–E.t.	67	29.07 ± 6.61	0.21 ± 0.51	0.198	NS	11	0.04 ± 0.01	0.27 ± 0.47	0.708	<0.05

locality, with the contribution of the same fungal species but involves a different host grass species.

In 2010, in the locality Pakość 1, 33 stromata of *E. typhina* from *D. glomerata* and 27 stromata of *Epichloë bromicola* from *Elymus repens* were collected to determine the effect of larval density on larval survival and weight. For each stroma the following parameters were determined: 1) size, 2) the number of live larvae, 3) the number of dead larvae, 4) the number of eggs before hatching, and 5) the number of eggs (unhatched eggs and larvae). Egg and larval density (per 1 mm^2), larval mortality rate (the ratio of dead larvae to the total number of larvae), and offspring success (the ratio of hatched eggs to the total number of eggs) were calculated. Live larvae were collected from stromata and weighed with an accuracy of 0.0001 g on a laboratory balance (A&D, HM-120).

Statistical Analyses. The correlation between a nominal variable (group affiliation) and quantitative variable (differences in egg density, offspring success, and larval weight, and the relationship between fly presence on a stroma and stroma length) was determined using either the *t*-test for independent variables, when two groups were compared, or one-way analysis of variance (ANOVA), when more than two groups were compared. If significant results were found, post hoc Tukey HSD tests were performed. Before the analyses were conducted, the assumptions of both tests were checked. The Kolmogorov–Smirnov test was performed to check normal distribution, while the Levene's test was used to check for a homogeneity of variance in groups. Welch's t-test was used when variations were not homogeneous. Pearson's correlation analysis was performed to check the relationship between 1) the number of eggs and stroma length, 2) offspring success and stroma length,

3) larval density and weight, and 4) larval density and mortality. All statistical analyses were conducted using Statistica v. 8.0 software (Statistica, Tulsa, OK).

Results

Stroma Size—Egg Number. In 2010, the number of fungal stromata with fly eggs differed significantly between the four studied grass–fungus associations ($\chi^2_3 = 224.5$; $P < 0.001$). The highest number of stromata with eggs (100%) was recorded in the population of *El. repens* infected with *E. bromicola* (El.r.–E.b.), while the lowest number of stromata with eggs was found in the population of *Holcus lanatus* (Table 2). The correlation between fly presence and stromata length was statistically significant only for the *P.d.–E.t.* association, in which insects more frequently visited longer stromata ($t_{438} = 6.04$; $P < 0.0001$; Table 2). A significant correlation between stroma length and number of eggs laid was found in the *P.d.–E.t.* ($r = 0.291$; $N = 440$; $P < 0.001$) and *El.r.–E.b.* ($r = 0.291$; $N = 54$; $P < 0.001$) associations (Table 3).

In long-standing observations of the infected populations of *P. distans*, significant differences in the number of stromata with eggs were found between individual years ($\chi^2_7 = 331.8$; $P < 0.001$). The percentage of stromata with eggs exceeded 50% only in one year (2003), while in other years, this value was highly variable, reaching its minimum in 2001 (1.36%) (Table 4). A significant positive connection between stroma length and fly presence was found in four out of eight years of observations (Table 4).The correlation between stroma length and egg number was significant in five out of eight years (Table 5).

In all studied vegetative seasons, except 2002, egg density differed significantly between the *E. typhina*

Table 4. Presence of *Botanophila* eggs on fungal stromata and the relationship between stroma length and egg presence in the *P. distans–E. typhina* association for the years 2000–2010

Year	N	Egg presence n (%)	Stroma lenght (mm) ± SD		P Levene's test	t	df	P
			Eggs present	No eggs				
2000	127	59 (46.46)	14.53 ± 2.82	11.19 ± 2.40	NS	7.21	125	<0.001
2001[a]	147	2 (1.36)	12.00 ± 2.83	15.63 ± 3.08	–	–	–	–
2002	120	8 (6.67)	13.75 ± 2.55	12.64 ± 2.77	NS	1.10	117	NS
2003	120	69 (57.50)	16.68 ± 3.09	16.02 ± 2.73	NS	1.20	118	NS
2006	60	7 (11.67)	13.14 ± 2.48	12.70 ± 2.63	NS	0.42	58	NS
2008	731	68 (9.3)	14.06 ± 3.29	12.46 ± 3.21	NS	3.85	729	<0.001
2009	592	74 (9.3)	13.45 ± 3.33	11.37 ± 2.83	NS	5.75	590	<0.001
2010	440	134 (30.45)	17.23 ± 3.85	14.85 ± 3.79	NS	6.04	438	<0.001
Overall	2,337	421 (18.01)	15.44 ± 3.71	12.85 ± 3.46	NS	13.71	2334	<0.001

[a] There were not enough cases in one group to conduct analysis.

Table 5. Correlation between stroma length and *Botanophila* egg number and between egg density and offspring success in the *P. distans–E. typhina* association for the years 2000–2010

Year	N	Stroma length ± SD	Egg no. ± SD	r	P	N	Egg density ± SD	Offspring success ± SD	R	P
2000	127	12.77 ± 3.09	0.76 ± 1.00	0.59	<0.001	59	0.11 ± 0.05	0.20 ± 0.38	−0.23	NS
2001	147	15.58 ± 3.10	0.01 ± 0.12	−0.14	NS	1	0.07 ± 0.00	0.00 ± 0.00	NS	NS
2002	119	12.71 ± 2.76	0.09 ± 0.37	0.03	NS	8	0.11 ± 0.06	0.25 ± 0.46	−0.44	NS
2003	120	16.41 ± 2.95	0.78 ± 0.81	0.19	<0.05	69	0.08 ± 0.03	0.58 ± 0.44	0.08	NS
2006	60	12.75 ± 2.59	0.13 ± 0.39	0.10	NS	7	0.09 ± 0.02	0.00 ± 0.00	NS	NS
2008	731	12.61 ± 3.25	0.09 ± 0.30	0.15	<0.001	68	0.08 ± 0.02	0.51 ± 0.50	0.027	NS
2009	592	11.63 ± 2.97	0.14 ± 0.40	0.21	<0.001	74	0.09 ± 0.05	0.45 ± 0.49	−0.16	NS
2010	440	15.57 ± 3.96	0.34 ± 0.56	0.29	<0.001	134	0.07 ± 0.02	0.70 ± 0.44	−0.07	NS
Overall	2,336	13.32 ± 3.65	0.22 ± 0.52	0.27	<0.001	420	0.08 ± 0.04	0.52 ± 0.48	−0.21	<0.001

stromata from two co-occurring populations, *P. distans* and *D. glomerata*, in the locality Pakość 1. Each year, the egg density was higher on the stromata from *D. glomerata* population (Table 6). In both associations, a significant relationship between the number of fly eggs and stroma length was found only in 2009; in this year, flies visited the longer stromata more frequently (Table 7).

Egg Density and Offspring Success. In 2010, the offspring success of *Botanophila* eggs on the stromata growing on *H. lanatus* (*H.l.–E.c.*) was significantly higher than with the other plant species ($F_{3.310} = 7.99$; $P < 0.001$). In both the *P.d.–E.t.* and *D.g.–E.t.* associations, no correlation was found between offspring success and egg density, while in the *El.r.–E.b.* association, offspring success increased as egg density decreased ($P = 0.708$; $N = 11$, $P < 0.05$; Table 3). Conversely, in the *H.l.–E.c.* association, offspring success increased with egg density ($P = 0.708$; $N = 11$, $P < 0.05$).

Offspring success in the populations of *P. distans* differed significantly between the years of observations ($F_{7,412} = 9.61$; $P < 0.001$). No significant correlation between egg density and offspring success in individual years was recorded (Table 5). However, when data from all years were considered the correlation was significant ($r = −0.21$; $N = 420$; $P < 0.001$). These data showed that the higher egg density, the less eggs hatch.

Offspring success differed significantly between the two co-occurring grass populations in the locality Pakość 1 in each studied growing season, except 2006. In the first two years, offspring success was higher in *D.g.–E.t.*, whereas in the last two years it was higher in *P.d.–E.t.* (Table 8). The correlation between offspring success and egg density in both associations in each year was not statistically significant, nor was the correlation significant between these parameters from the overall data from all the years of the study (Table 9).

Table 6. Differences in *Botanophila* egg density on fungal stromata in two associations (*P. distans–E. typhina* and *D. glomerata–E. typhina*) that co-occur in the locality

Year	Egg density ± SD		P Levene's test	t	df	P
	P.d.–E.t.	*D.g.–E.t.*				
2002	0.004 ± 0.016	0.010 ± 0.031	NS	−0.96	58	NS
2003	0.056 ± 0.049	0.102 ± 0.052	NS	−3.59	58	<0.001
2006	0.008 ± 0.025	0.074 ± 0.058	<0.001	−7.26	79.35	<0.001
2008	0.017 ± 0.034	0.045 ± 0.032	NS	−6.75	268	<0.001
2009	0.010 ± 0.027	0.022 ± 0.022	NS	−2.29	177	<0.05
Overall	0.016 ± 0.033	0.051 ± 0.048	<0.001	−10.37	450.42	<0.001

Table 7. Relationship between stroma length and *Botanophila* egg presence in two associations (*P. distans–E. typhina* and *D. glomerata–E. typhina*) that co-occur in the locality Pakość 1

Grass–fungus association	Year	Stroma lenght (mm) ± SD		P Levene's test	t	df	P
		Eggs present	No eggs				
P.d.–E.t.	2002	15.50 ± 0.71	13.18 ± 2.34	NS	NS	NS	NS
	2003	16.00 ± 2.64	16.80 ± 3.22	NS	0.73	28	NS
	2006	12.67 ± 2.08	11.96 ± 2.05	NS	−0.56	28	NS
	2008	13.75 ± 3.18	12.69 ± 2.71	NS	−1.86	141	NS
	2009	13.53 ± 2.20	11.27 ± 2.49	NS	−3.75	148	<0.001
	Overall	14.29 ± 2.90	12.20 ± 2.77	NS	−5.84	381	<0.001
D.g.–E.t.	2002	60.62 ± 21.02	54.59 ± 16.82	NS	−0.81	28	NS
	2003	–	41.27 ± 9.34	NS	NS	NS	NS
	2006	45.10 ± 12.43	53.25 ± 6.70	NS	1.29	53	NS
	2008	54.76 ± 13.46	51.58 ± 9.41	NS	−0.80	125	NS
	2009	41.63 ± 11.67	31.50 ± 7.60	NS	−2.47	27	<0.05
	Overall	49.82 ± 14.20	48.91 ± 15.62	NS	−0.39	269	NS

Table 8. Differences in offspring success on fungal stromata in two associations (*P. distans–E. typhina* and *D. glomerata–E. typhina*) that co-occur in the locality Pakość 1

| Year | Offspring success ± SD | | *P* Levene's test | *t* | df | *P* |
	P.d.–E.t.	*D.g.–E.t.*				
*2002	0.50 ± 0.71	1.00 ± 0.00	–	NS	NS	NS
2003	0.50 ± 0.43	0.74 ± 0.28	<0.05	−2.20	29.62	<0.05
2006	0.00 ± 0.00	0.48 ± 0.36	<0.05	−9.59	50.00	<0.001
2008	0.66 ± 0.48	0.61 ± 0.41	<0.05	0.51	43.97	NS
2009	0.84 ± 0.37	0.23 ± 0.42	NS	4.78	36	<0.001
overall	0.63 ± 0.46	0.58 ± 0.40	<0.001	0.96	297	NS

*, not enough valid cases in one group to conduct the analysis.

Density of Larvae and Their Survival and Weight. The mean weights of larvae collected from the *E. typhina* stromata occurring on *D. glomerata* (*D.g.–E.t.*) and the *E. bromicola* stromata on *El. repens* (*El.r.–E.b.*) did not differ ($t_{48.78} = 1.77$; $P = 0.083$) in both grass–fungus associations and were 0.0032 ± 0.0016 g and 0.0026 ± 0.0009 g, respectively. No significant correlations between larval density on a stroma and either larval weight or mortality were observed. In both cases, offspring success was correlated negatively with egg density on a stroma ($r = -0.3997$; $N = 33$; $P < 0.05$ for *D.g.–E.t.*, and $r = -0.4880$; $N = 27$; $P < 0.01$ for *El.r.–E.b.*).

Discussion

Botanophila flies use stromata of *Epichloë* fungi for laying their eggs and for larval feeding and development. Larvae feed on the stromata and, after metamorphosis, they overwinter in the soil. It seemed highly likely that a fly should choose a larger stroma on which to lay its eggs because a larger stroma would assure more food to the offspring of the fly. However, the results of this study do not confirm this assumption unambiguously. In 2010, flies chose longer stromata only in two examined grass–fungus associations (*El.r.–E.b.* and *P.d.–E.t.*) out of four. In these two associations, egg density (excluding stromata with no eggs) was higher than in the other two associations (*H.l.–E.c.* and *D.g.–E.t.*). In the latter associations, contrary to our expectations, flies showed no preference for longer stromata. It also seems that egg density on a stroma

does not affect offspring success. A negative correlation between egg density and offspring success was observed in only one association (*El.r.–E.b.*). Moreover, the percentage of hatched eggs in this association did not differ significantly from that found in the association with the lower egg density, *D.g.–E.t.*, in which flies showed no preference for larger stromata.

The above results question our presumption that egg laying depends on stroma length and offspring success on egg density. However, long-term (2000–2010) observations of the fly–fungus interaction in the *P.d.–E.t.* association showed that the fly preference for stromata of different sizes might change in time. Although the general data clearly indicate that flies prefer longer stromata, an analysis of fly behavior in consecutive years revealed that in one year they may choose longer stromata, while in the next, they show no preferences.

There may be different reasons for this lack of fly preference for stromata size in different grass–fungus associations. The grasses *H. lanatus* and *D. glomerata* grow sparsely, and their tussocks with stromata sometimes grow at a considerable distance from each other, unlike *El. repens* or *P. distans.* It is likely that when the distance between plants with stromata is large, a fly lays its eggs on the nearest available stroma. Conversely, when plants with stromata grow in high density, an insect chooses the longest stromata. Such preference (or lack of preference) may also be correlated with the mean length of fungal stromata occurring on individual grass species. If this value is low, as in *E. typhina* on *P. distans*, flies may omit the shortest stro-

Table 9. Correlation between *Botanophila* egg density and offspring success in two associations (*P. distans–E. typhina* and *D. glomerata–E. typhina*) that co-occur in the locality Pakość 1

Grass–fungus association	Year	Offspring success ± SD	Egg density ± SD	*N*	*r*	*P*
P.d.–E.t.	2002	0.50 ± 0.71	0.06 ± 0.003	2	NS	NS
	2003	0.50 ± 0.43	0.08 ± 0.03	20	0.12	NS
	2006	0.00 ± 0.00	0.08 ± 0.01	3	NS	NS
	2008	0.66 ± 0.48	0.08 ± 0.02	32	−0.15	NS
	2009	0.84 ± 0.37	0.08 ± 0.02	19	−0.34	NS
	Overall	0.63 ± 0.46	0.08 ± 0.02	76	−0.08	NS
D.g.–NSE.t.	2002	1.00 ± 0.00	0.04 ± 0.05	8	NS	NS
	2003	0.74 ± 0.28	0.10 ± 0.05	30	−0.11	NS
	2006	0.48 ± 0.36	0.08 ± 0.06	51	−0.12	NS
	2008	0.61 ± 0.41	0.05 ± 0.03	115	0.07	NS
	2009	0.23 ± 0.42	0.03 ± 0.02	19	−0.29	NS
	Overall	0.58 ± 0.40	0.06 ± 0.05	223	0.01	NS

mata that are likely to be too small to ensure a sufficient food supply for two, or even for one, larva.

The lack of unambiguous correlation between fly preference for a stroma and stroma length may also result from the lack of distinct relationships between egg density and offspring success. Such a connection was only observed in a few cases; thus, searching for longer stromata, which are also attractive to other female flies, can be an unnecessary energy expense. This may explain why offspring success was the same for long and short stromata. It is also possible that *Botanophila* females make a choice based on other stromata traits, such as volatile substances. Odor profiles of fungal stromata exhibit considerable variation, although profiles among species overlap and may not discriminate at the species level (Steinebrunner et al. 2008a). It is possible that the varied intensity of smell may determine the attractiveness of stromata to female flies. However, the latter thesis would require evidence that there is no correlation between the amount of volatile substances produced and stroma length.

An analysis of fly preferences for stromata was conducted by Pawlitz and Bultman (2000). It involved such traits as stroma size, distance of stromata from the ground and their density on a single grass tussock, as well as tussock density. According to these authors, fly preferences depend not only on a fungal species but also on the presence or absence of another, alternative fungal host species in a given area. When only one species was available, a fly chose longer stromata. Conversely, when another host species co-occurred in the area, a fly showed no preference for stroma length. These results partially coincide with our results for the co-occurring populations of *P. distans* and *D. glomerata* in the locality Pakość 1. Although both grasses are infected with the same fungal species (*E. typhina*), they differ in length of associated stromata. In *D. glomerata*, the length of stromata is four times greater than in *P. distans.* The relationship between fly presence and stroma length in the discussed population of *P. distans* was found in only one growing season (the last year of this study), while in the populations that occur individually, such a relationship was observed in each season.

A high density of eggs on a single stroma may theoretically be a cause of food deficiency for the subsequent larvae. However, the results of our investigations conducted in two associations, *D.g.–E.t.* and *El.r.–E.b.*, do not confirm this assumption; the weight of larvae did not depend on their density on a stroma. Similar results were previously obtained by Rao and Baumann (2004). In addition, the mortality of larvae in the studied grass–fungus associations was not correlated positively with larval density on a stroma, as opposed to the results of previous studies (Bultman et al. 2000, Pawlitz and Bultman 2000). Although single dead larvae were observed inside their cocoons, there was no food shortage, as feeding traces of larvae did not overlap. In addition, neither empty cocoons, which would suggest a predator attack, nor fungal infections were observed. These results suggest that another factor is responsible for larval mortality.

In summary, the results of this study do not allow us to unambiguously determine the preferences of *Botanophila* flies for stroma length during their search for a suitable place to lay their eggs. Some differences were found between the individual grass–fungus associations, while long-term observations showed that fly preferences could vary in time with no clear tendencies. In addition, the effect of weather conditions on the presence or absence of such preferences cannot be excluded. First, if weather conditions determine the time of metamorphosis and its success, the total number of female flies that occur within a given grass–fungus association may fluctuate, which can affect the proportion of females to fungal stromata in a given area. Second, if the number of stromata is too small in relation to the number of females, finding any place to lay eggs is a priority, regardless of stroma length. The observed lack of an effect of egg and larval density on the fate of offspring indicates that other attributes of stromata may be of importance. Previous studies showed that in the case of herbivorous insects, their preferences in the selection of places to lay eggs were not always correlated with higher survival rates (Courtney and Kibota 1990, Zimmerman and Brody 1998, Harris et al. 2001). A similar situation may occur in the *Botanophila–Epichloë* interaction.

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