Spore dissemination by mycophagous adult drosophilids

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Abstract

Although most fungal spores are transported by wind, some remain on lamellae even after fungal fruiting bodies start to decay. This raises the question: are these remaining spores useless or instead transported by other means? In this study, we accordingly investigated whether adult dipteran insects transport fungal spores. Our examination revealed that fungal spores were present in intestines of most drosophilid adults but almost completely absent from those of other dipteran adults. At least some spores excreted by *Drosophila angularis* and *D. brachynephros* retained the capacity to germinate. Structural damage to spores that passed through the digestive tract of these two drosophilid species varied among fungal species, with a greater number of colorless spores generally damaged than colored ones. These results suggest that adult drosophilid flies, but not other dipterans, can transport fungal spores.

Keywords: Basydiomycota, Fungal reproduction, Endozoochory, Diptera, *quinaria* species group

Introduction

Most fungal species disperse their spores by wind, with their fruiting bodies usually possessing morphological structures to efficiently release spores into the air (Ingold 1971). Examples include the fruiting bodies of basidiomycete fungi (i.e. mushrooms or toadstools). Because fungal fruiting bodies are often nutrition-rich, however, they serve as food for a number of animal and insect species. As a result, fungi have evolved various defense measures, such as toxic chemicals, to avoid attacks by animals or insects. In addition, some fungi exploit animals and insects as spore dispersal agents (Buller 1909; Ingold 1971; Malloch and Blackwell 1992). For example, stinkhorn mushrooms attract flies and some other insects by emitting a foul-smelling odor and use these insects to disperse their spores (Ingold 1971; Malloch and Blackwell 1992; Tuno 1998). In addition, spores of the bracket fungus *Ganoderma applanatum* that have passed through the intestines of one drosophilid species have been found to maintain germination capacity, which suggests that this drosophilid acts as a spore dispersal agent (Tuno 1999). Likewise, Lilleskov and Bruns (2005) have concluded that some soil animals and insects act as spore dispersers of *Tomentella sublilacina*, as the spores of this resupinate ectomycorrhizal fungus are resistant to ingestion by those animals and insects and also adhere to their body surfaces. Despite these findings, studies on spore dispersion by animals and insects are still limited.

The aim of this study was to determine whether dipteran insects, particularly drosophilid flies, can act as spore dispersers. Although some bark and ambrosia beetles have evolved specialized organs called mycangia for transportation of spores, most insects, including dipteran insects, transport spores on their legs or bodies or in their intestines (Ingold 1971; Malloch and Blackwell 1992). In this study, we focused on spore transportation via intestines. For spores to be transported in intestines, they must be consumed by insects and be resistant to digestive enzymes. We searched for fungal spores in intestines of adult dipteran insects collected from fungal fruiting bodies in the field.

We then looked for structural damage to spores that had passed through the intestines of drosophilid flies and also tested their germination capacity.

Materials and Methods

Liberation of spores

Fungal fruiting bodies were collected from a broad-leaved deciduous forest on the Kakuma campus of Kanazawa University in 2014. Fruiting bodies were classified into three groups: young bodies with non-fully opened caps, mature ones with fully opened caps, and old ones beginning to decay. To examine spore liberation, fungal caps were removed from stipes, placed on Petri dishes (for fungal species with colorless spores) or powder paper (for those with colored spores), covered with plastic cups and left for 24 h. If spores were liberated, spore prints were obtained. If spore prints were not generated, this indicated that spores were either absent or tightly adhered to lamellae. To distinguish between these two possibilities, lamellae on old fruiting bodies not yielding spore prints were examined for the presence/absence of spores. Fungal caps were rinsed in 50 mL water; 20 µL of the resulting solution was transferred to a hemocytometer, and the number of spores was counted under a microscope.

Spore presence/absence in the intestines of wild-caught insects

Adult insects collected from fruiting bodies with an aspirator from June 21 to November 1 in 2013 in the Kakuma forest were preserved in 70% alcohol for identification. Drosophilid flies were identified to species, whereas other dipteran individuals were identified to genus or family. After identification, the insects were dissected and their intestines were examined for spores under a microscope. Because only a few individuals from Phoridae (genus *Megaselia*), Mycetophilidae, Cecidomyiidae, Sciaridae, Heleomyzidae (genus *Suillia*), and Chloropidae were present in the 2013 samples, individuals from these families collected in 2012 and 2014 from the Kakuma forest were also examined.

Excretion of spores by drosophilid flies

Excretion of spores from two *Drosophila* species, *D. angularis* Okada and *D. brachynephros* Okada, was investigated using *Coprinus atramentarius* (Bulliard ex Fries) as a spore source. Laboratory stocks of these *Drosophila* species were obtained from Ehime University (*D. angularis*: E-16901FK05-14; *D. brachynephros*: E-16501MTY04). Spore prints were prepared by placing *C. atramentarius* caps on 2% agar plates in Petri dishes (9 cm diameter; 1.5 cm depth) for 1 h. Three- to five-day-old adult flies, which were starved for a day in vials containing only wet tissue paper to facilitate feeding, were placed in Petri dishes with spore prints and allowed to ingest spores for a day. Individual flies were then placed in empty Petri dishes along with a piece of wet cotton and kept at 22°C under long-daylength conditions (15 h light/9 h dark). Flies began excretion immediately after introduction into dishes and continued until they died. Excrements were color-labeled 1, 2, 3, 4, 8 and 12 h after the introduction of flies and every 12 h thereafter. Each excrement was transferred into a 200-µL tube via a micropipette and homogenized in 30 µL of 5% SDS solution. The excrement solution was vortexed, and the spore density of a 20-µL aliquot was determined using a hemocytometer.

Germination of spores

Spore germination was examined in three fungal species collected from the Kakuma forest: *Pleurotus djamor* (Rumphius ex. Fries), *Coprinus micaceus* (Bulliard ex Fries) and *Naematoloma sublateritium* (Fries). To prepare intact spores, mushroom caps were placed on plates containing potato dextrose agar (PDA) or 2% agar supplemented with benomyl (10 mg/L) as an antifungal agent, covered with plastic cases, and left for a few hours. When enough spores had fallen on the plates, the plates were sealed with polyethylene film and kept at 22°C under long-daylength conditions. To obtain spores that had passed through the digestive tract of *D. angularis* or *D. brachynephros*,

starved adult flies, prepared as above, were fed spores by placing the insects directly on mushrooms or on plates with spore prints prepared as above. When flies started excretion, they were individually placed in Petri dishes with 2% agar plates without added nutrients. Excrements were collected using micropipettes as described above and dissolved in sterile water. Medium (20 µL) with excrements was dripped onto PDA plates (2% agar) or 2% agar plates and expanded using a Conradi rod. The plates were sealed and kept at 22°C under long-daylength conditions. Spores on the culture plates were observed under a microscope, and the proportion of germinated spores in a microscopic field (3.80 mm^2 visual field) was recorded. One to five culture plates were prepared for each condition, with each plate examined once or twice over 2–7 days after the start of culturing. The germination rate at each observation was determined by averaging the proportions in eight microscopic fields.

Damage to spores

For various reasons, fungal spores do not always germinate on agar or PDA plates. For instance, ungerminated spores may be damaged or in dormancy. In addition, the culturing conditions may not be appropriate for their germination. In this study, we therefore checked for structural damage to spores that passed through drosophilid digestive tracts. Eleven fungal species, namely, *Hygrocybe conica*, *Russula flavida*, *Amanita vaginata*, *Inocybe* sp., *Entoloma kujuense*, *Coprinopsis atramentaria*, *Hypholoma lateritium*, *Suillus granulatus*, *Cortinarius purpurascens*, *Boletellus floriformis* and *Agaricus* sp., were collected from the Kakuma forest. These species were identified on the basis of morphological characteristics according to Ikeda (2013). Medium containing spores that had passed through the digestive tract of *D. angularis* or *D. brachynephros* was prepared as described above, placed on a glass slide, and inspected for damaged spores under a microscope. Spores lacking oil drops were considered to be damaged (Nakamori and Suzuki 2010). Approximately 300 spores were observed. The damage rate (DR) was determined by the following formula (Nakamori and Suzuki 2010):

$$DR = 100 \times (a - b)/(c - b),$$

where a is the number of spores without oil drops, b is the number of control (intact) spores without oil drops, and c is the total number of observed spores. Spores collected from spore prints were used as a control.

Statistical analyses

Statistical analyses were performed using JMP ver. 5.0.1 (SAS Institute, Cary, NC, USA).

Results

Liberation of spores

A total of 129 (10 young, 65 mature and 54 old) fruiting bodies representing eight fungal species were collected. Of these, spore prints were not obtained from 4 (40%) young, 14 (22%) mature and 21 (39%) old fruiting bodies (Fig. 1). The difference in the frequency of non-generated spore prints among the three age groups was not significant (χ^2 test, $P = 0.095$). Eleven of the 14 mature fruiting bodies that failed to yield spore prints were *Gymnopus peronatus*. Seventeen of the 21 old fruiting bodies not yielding spore prints were checked for spores on lamellae (Table 1): spores were present in 11 fruiting bodies, but none were observed in any of the five fruiting bodies of *G. peronatus* nor in one fruiting body of *Amanita citrina*.

Spores in intestines of wild-caught insects

In total, 257 dipteran adults from 14 families were collected in 2013 from 20 fungal species

belonging to seven families. Drosophilid flies represented 63% of dipteran insects, with three *Drosophila* species, *D. angularis, D. brachynephros* and *D. bizonata*, comprising 92% of drosophilids (Table 2). The intestines of 248 insects collected in 2012, 2013 and 2014 were examined for fungal spores. Fungal spores were present in 81% of examined drosophilid flies (Table 2). Crops of most of these individuals contained many spores (usually 10^4 to 10^6), with some containing spores of more than one species (data not shown). With respect to insects from other families, one individual, belonging to Phoridae (*Megaselia* sp.), contained a few spores. This individual was collected from rotten *Phylloporus bellus* and may have accidentally ingested spores remaining on the fruiting body.

Excretion of spores by drosophilid flies

Spore-fed drosophilid flies excreted numerous spores within 12 h of feeding (Table 3). *Drosophila angularis* females excreted, on average, 22,544 spores per excrement, while *D. angularis* males excreted 14,814 spores and *D. brachynephros* males passed 12,766. The maximum number of spores observed in an excrement of a *D. angularis* female, *D. angularis* male and *D. brachynephros* male was 93,600, 52,800 and 131,400, respectively. The number of spores in excrements decreased drastically after 12 h.

Germination of spores

Spores began to germinate within a day of the start of culturing (data not shown). In this study, the germination rate was determined 2–7 days after the start of culturing. The germination rate of intact spores was 55.5% in *Pleurotus djamor*, 18.0% in *Coprinus micaceus* and 7.3 % in *Naematoloma sublateritium*; in contrast, the germination rate of spores after passage through the digestive tract of drosophilid flies was generally lower, i.e. 3.8–9.7% (Table 4).

Damage to spores

The fungal species examined in this study differed in spore coloration: spores of *Hygrocybe conica*, *Russula flavida* and *Amanita vaginata* were colorless, whereas those of the others were more or less colored. The frequency of damaged spores considerably varied among fungal species (Fig. 2). A high percentage (> 90%) of colorless spores were damaged. Colored spores, other than those of *Inocybe* sp. (78%), were less frequently damaged.

Discussion

In this study, mature fruiting bodies of most fungal species usually produced spore prints. One exception was *Gymnopus peronatus*, which seldom produced prints. In addition, fruiting bodies of *G. peronatus*, at least old ones, did not appear to retain spores on lamellae. Fruiting bodies of *G. peronatus* thus may not produce any basidiospores. Determining why the spore production of this species differs from that of other fungi is an interesting question.

Noteworthily, old fruiting bodies of most fungal species retained spores on hymenophores (lamellae or tubes) even though they produced no spore prints or only faint ones. This observation suggests that not all of their spores are released into the air. As discussed below, animals or insects may contribute to dispersal of such unreleased spores.

In the present study, 81% of adult drosophilid flies collected from fungal fruiting bodies had fungal spores in their intestines, whereas other dipteran insects contained almost no fungal spores. This result suggests that non-drosophilid dipteran species rarely feed on spores. In previous field studies, more than 60% of dipteran individuals emerging from mushrooms were non-drosophilids (Toda et al. 1999; Kobayashi et al. unpublished data); in the present study, in contrast, non-drosophilid dipteran adults constituted less than 50% of all dipteran adult individuals collected from fungal fruiting bodies. These results suggest that non-drosophilid dipteran adults do not feed on spores, but instead visit fungal fruiting bodies mostly for oviposition. The exceptions are insects

attracted to fruiting bodies of *Phallus indusiatus* (Phallales: Phallaceae); they feed on spores in gleba (Tuno 1998). However, these insects are fruit-feeders, not true fungus-feeders, and *P. indusiatus* fruiting bodies emit an odor similar to fallen fruits to recruit fruit-feeding insects as spore dispersers (Tuno 1998). The present results further suggest that only drosophilid flies transport spores in their intestines; however, other dipteran species as well as drosophilid flies may transport spores on legs or bodies, as indicated in a study on *Ganoderma applanatum* (Tuno 1999).

Fungal spores that passed through intestines of drosophilid flies were more or less damaged. The frequency of damage differed by spore type: more than 90% of colorless spores were damaged, with colored spores generally exhibiting less damage. Cell walls of colorless basidiospores consist of one or two layers, whereas those of colored spores comprise three to six layers (Furukawa 1992). The thick cell walls of colored spores may be more resistant to insect digestive enzymes. In fact, *Ganoderma* spores have very thick cell walls, and only the outer layers of their walls are lightly damaged even when passed through intestines of drosophilid flies (Tuno 1999). In our experiments, however, colorless spores of *Pleurotus djamor* and colored spores of *Coprinus micaceus* or *Naematoloma sublateritium* did not differ substantially in germination rate after passage though digestive tracts of drosophilid flies. Further study is needed on the resistance of spores to digestive enzymes.

The large number of spores ingested by flies may compensate for the high frequency of damaged spores emerging from the digestive tract. In this study, almost 100,000 spores were contained in excrements of drosophilid flies. If even 99% of these spores were damaged, 1,000 would still be alive. Furthermore, transportation of spores by animals or insects may be advantageous for colony formation. According to one report, colony formation does not occur in some ectomycorrhizal fungi when spore density is low (less than 10^3) and varies depending on the density of spores (between 10^3–10^6 spores per seedling) in those species (Rincón et al. 2001). Such highly aggregated distribution of spores would rarely occur via wind dispersal. For example, Li (2005)

showed in his study on *Amanita muscaria* var. *alba* that the average density of spores was 7,137/m^2 at a distance of 0.3 m from the fruiting body but only 940/m^2 at a distance of 5.2 m. Galante et al. (2011) have also reported that 95% of spores released into the air land within 1 m of the fruiting body, with the number of fallen spores decreasing exponentially with distance from the fruiting body. In contrast, high-density patches could occur if spores are carried by animals, e.g. drosophilid flies. Many fungal species start to decay in a relatively short time after emergence (Ingold 1971), a possible strategy to attract flies for transportation of spores.

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Figure legends

Fig. 1. Proportion (in black) of fungal fruiting bodies (caps) of young, mature and old mushrooms that failed to yield spore prints. Numbers above columns indicate the number of examined fruiting bodies (caps). The difference in frequency among the three age groups was not significant ($\chi 2$ test, $P = 0.095$).

Fig. 2. Proportion of spores exhibiting damage after passage through the digestive tract of *Drosophila angularis* or *D. brachynephros*. Spores from the following species were examined: *Hygrocybe conia* (Hc), *Russula flavida* (Rf), *Amanita vaginata* (Am), *Inocybe* sp. (Is), *Entoloma kujuense* (Ek), *Coprinopsis atramentaria* (Ca), *Hypholoma lateritium* (Hl), *Suillus granulatus* (Sg), *Cortinarius purpurascens* (Cp), *Boletellus floriformis* (Bf) and *Agaricus* sp. (As). White and black columns correspond to fungal species with colorless and colored spores, respectively. Five females of each *Drosophila* species were used, with approximately 300 spores examined per excrement.

Table 1. Estimated number ($\times 10^6$) of spores per fruiting body observed in "old" fruiting bodies that failed to yield spore prints.

Mushroom species	*N*	Range
Tylopilus fumosipes	1	54.2
Pulveroboletus ravenelii	1	43.8
Amanita citrina var. *citrina*	2	0-4.1
Amanita vaginata	1	93.3
Lactarius gracilis	3	6.5-25.6
Russula bella	2	51.8-78.7
Russula sp.	2	*
Gymnopus peronatus	5	0

* Spores were present but not counted.

Table 2. Number of adult individuals collected in 2013, the number examined for spores in intestines, and the number with detectable spores.

Dipteran family	Collected	Examined	With spores present
Drosophilidae	163	134	109
Phoridae	46	36*	1
Sphaeroceridae	17	17	0
Mycetophilidae	1	15*	0
Cecidomyiidae	10	13*	0
Sciaridae	5	8*	0
Heleomyzidae	1	8*	0
Chloropidae	4	7*	0
Ceratopogonidae	4	4	0
Chironomidae	2	2	0
Milichiidae	1	1	0
Dolichopodidae	1	1	0
Platypezidae	1	1	0
Clusiidae	1	1	0

* Includes additional individuals collected in 2012 and 2014.

Table 3. Number of spores (mean ± SD) present in an excrement of *Drosophila angularis* or *D. brachynephros*. Excrements were collected 12-96 h after spore ingestion.

	Drosophila angularis		*D. brachynephros*
Time (h)	Females (7)	Males (7)	Males (7)
12	22,544 ± 5,118	14,814 ± 5,931	12,766 ± 9,319
24	4,943 ± 6,007	4,170 ± 4,135	35 ± 125
36	7 ± 11	1337 ± 31	27 ± 87
48	4 ± 5	76 ± 197	63 ± 128
60	0	0	2 ± 6
72	19	0	226 ± 203
84	12	0	0
96	38	0	0

The number of individuals used in the experiment is indicated in parentheses.

Table 4. Germination rates (%) of control spores and spores passed through the digestive tract of *Drosophila angularis* or *D.brachynephros*. Germination rates were examined 2-7 days after the start of culturing.

Mushroom species	Control	*Drosophila angularis*	*D. brachynephros*
Pleurotus djamor	55.5 (1, 2)	5.2 (1, 1)	-
Coprinus micaceus	18.0 (1, 2)	8.3 (5, 1)	9.7 (2, 1)
Naematoloma sublateritium	7.3 (1, 2)	-	3.8 (2, 1)

The two numbers within each set of parentheses respectively indicate the number of prepared plates and the number of times the plates were observed.

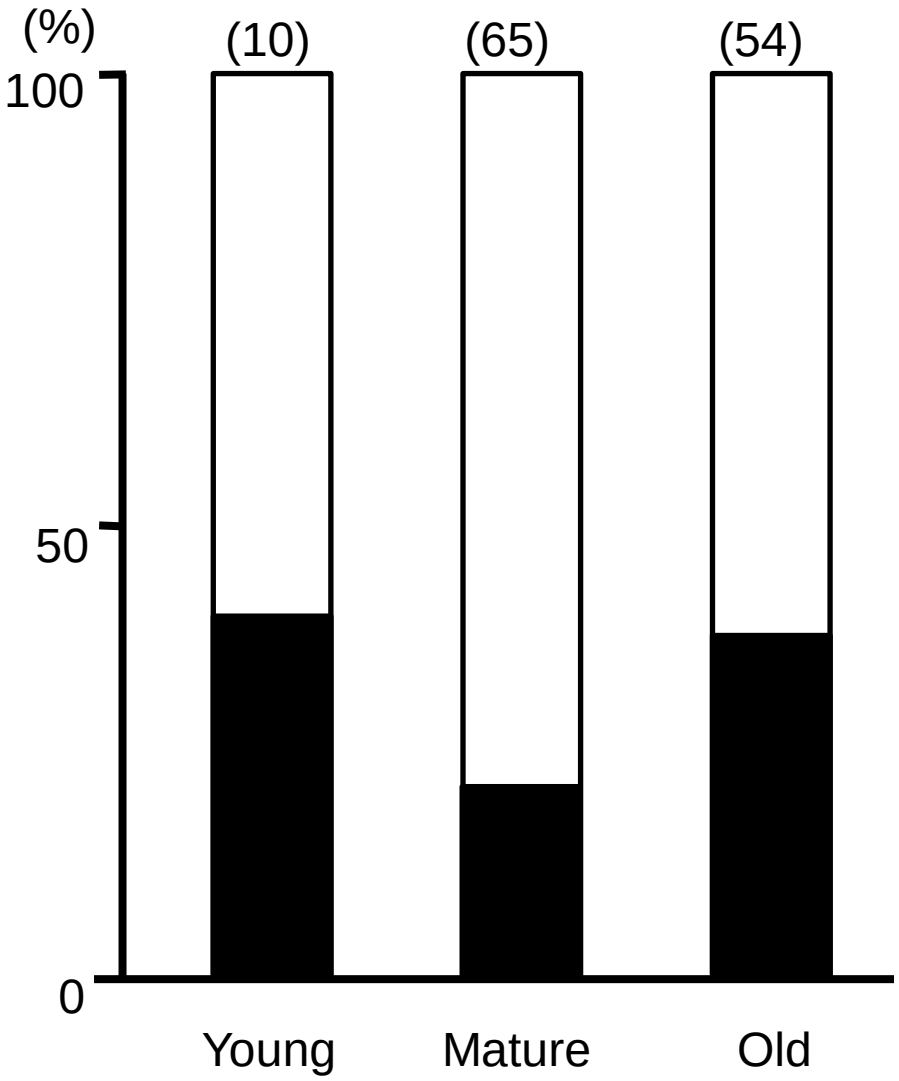
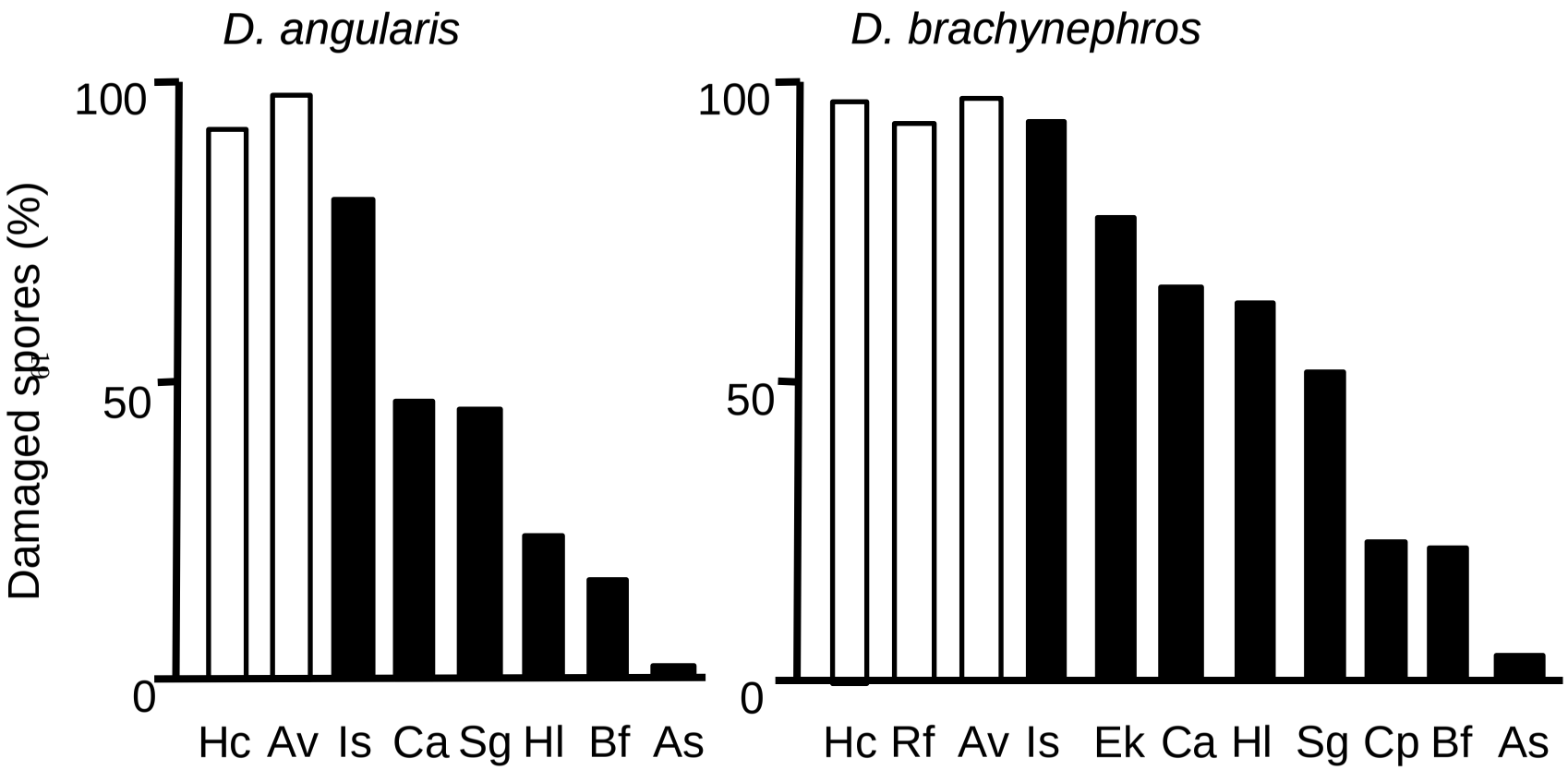


Fig. 1


D. angularis
D. brachynephros
Damaged spores (%)
100
50
0
Hc Av Is Ca Sg Hl Bf As
100
50
0
Hc Rf Av Is Ek Ca Hl Sg Cp Bf As