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Defensive role of cystidia against *Collembola* in the basidiomycetes *Russula bella* and *Strobilurus ohshimae*

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ABSTRACT

Cystidia in fruit bodies are taxonomically important characters. However, little is known about their ecological functions. The defensive role of cystidia against the collembolans *Ceratophysella denisana* and *Mitchellania horrida* was examined in fruit bodies of *Russula bella* and *Strobilurus ohshimae*. Cystidium-destruction experiments demonstrated that *R. bella* and *S. ohshimae* cystidia decrease the number of collembola found on gills, although the effects were not significant for *R. bella* against *C. denisana*. Furthermore, *R. bella* cystidia increased collembolan mortality in the laboratory, and in the field, collembola were found dead on parts of the fruit body of this species where cystidia were abundant. In the cystidium-destruction experiment, approximately one-third of collembola appeared to avoid *R. bella*. Therefore, deadly cystidia may be selected for in *R. bella* to avoid collembolan attack. Laboratory feeding experiments revealed that collembola can extensively damage *R. bella* and *S. ohshimae* basidiospores by feeding. These results suggest that the cystidia of *R. bella* and *S. ohshimae* may protect basidiospores from collembolan predation.

Introduction

Collembola feed on the mycelia and fruit bodies of many fungi. Fungi have various strategies for coping with collembolan grazing; e.g. some fungi exhibit compensatory growth in response to grazing (Hedlund *et al.* 1991; Kampichler *et al.* 2004). Some fungi are thought to produce toxins or other secondary metabolites to protect themselves from fungivores, including collembola (Shaw 1988). The consumption of fungal melanin reduces the growth and reproductive rates of collembola (Scheu & Simmerling 2004).

Collembola are common mushroom feeders (Greenslade *et al.* 2002; Nakamori & Suzuki 2005a; Sawahata *et al.* 2000) that can aggregate by the thousands on a single fruit body (Sawahata *et al.* 2000) and may reduce the number of wind-dispersed spores (Sawahata 2006). Although collembola are thought to be generalist feeders, certain fungal species are rarely attacked (Mateos *et al.* 1996), suggesting the existence of fungal defence systems. Some studies have examined the defences of fruit bodies against other fungivores, including flies, slugs, and opossums (Bruns 1984; Camazine 1983; Camazine *et al.* 1983; Stadler & Sterner 1998; Sterner *et al.* 1985; Wood *et al.* 2001, Wood *et al.* 2004). However, fungal defences against collembola have received little attention (Nakamori & Suzuki 2006).

Certain species of fungi have cystidia that project from the gills. Although cystidia are an important taxonomic character, their function remains unclear. Among several possible functions (Largent *et al.* 1977), cystidia may protect fruit bodies from predators (Buller 1909), although they do not protect

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certain species from slugs (Buller 1922). To our knowledge, the defensive role of cystidia against other animals has not been investigated. Cystidia may be involved in resistance to grazing by micro- and mesofauna, such as collembola, that are adversely affected by the microscopic structures of fungi and plants (Drechsler 1944; Turnipseed 1977).

To test the hypothesis that cystidia protect basidiospores from predation by collembola, we examined effects of cystidia on collembolan survival and the number of collembola found on the gills of two cystidium-bearing fungi, *Russula bella* and *Strobilurus ohshimae*, in the laboratory. We also observed collembolan death on *R. bella* fruit bodies in the field, and examined the sensitivity of basidiospores to feeding by collembola.

Materials and methods

Microscopic observations of intact Russula bella *cystidia*

Cystidia were placed on glass slides and observed under a microscope without mounting medium or a coverslip.

Location of dead collembola on Russula bella *fruit bodies in the field*

To determine the location of dead collembola on fruit bodies in the field, *Russula bella* fruit bodies were collected on five occasions in 2003 in Minamisaku, Nagano, Japan (35°57′ N, 138°28′ E; $n = 16$), and collembolan death was evaluated on-site. Animals that did not move, even when stimulated by air blows, were considered dead. Three fruit bodies of *R. violeipes* with dead collembola from Chiba, Japan (35°34′ N, 140°13′ E), were examined on 5 July 2003.

Cystidium-destruction experiment

To examine the effects of *Russula bella* and *Strobilurus ohshimae* cystidia on collembolan survival and number on fruit bodies, collembola were presented with fruit bodies with intact or destroyed (see below) cystidia in the laboratory. In *R. bella*, cystidia are dense on the cap (pileocystidia), stipe (caulocystida), and the edges of gills (cheilocystidia), whereas cystidia are sparse on the sides of gills (pleurocystidia; Hongo 1968). The fruit bodies of *S. ohshimae* are entirely covered with cystidia (Hongo 1955).

The collembola *Ceratophysella denisana* and *Mitchellania horrida* were selected because dead specimens of these species were recorded on field-collected *R. bella* (see Results), and they are common on fruit bodies (Fjellberg 1985; Nakamori & Suzuki 2005a). Experiments were conducted for *R. bella* against both *C. denisana* and *M. horrida*, and for *S. ohshimae* against *C. denisana*. *C. denisana* specimens for the *R. bella* test were collected from Minamisaku and starved for 3 d on a culture substrate (moist mixture of plaster of Paris and activated charcoal) at 23 °C before use. *M. horrida* specimens were collected from Chiba; they were fed tissues of *Pholiota nameko* and then starved for 10 d on culture substrate at 23 °C before use. *C. denisana* specimens for the test on *S. ohshimae* were collected from Otsu, Shiga, Japan (34°56′ N, 135°53′ E), fed *P. nameko* and then starved for 7 d on culture substrate at 20 °C before use. Fruit bodies of *R. bella* were collected from Minamisaku and stored at 8 °C for 4 or 1 d before use in tests with *C. denisana* or *M. horrida*, respectively. Only the base of the stipe was touched when fruit bodies were collected. Fruit bodies of *S. ohshimae* were obtained from laboratory cultures on *Cryptomeria japonica* twigs that were collected from Chiba and maintained at *ca* 15 °C under a 12:12 light:dark photoperiod in the laboratory, according to Homma *et al.* (2005). The bases of *S. ohshimae* stipes were cut to 1–2 cm length, and the cut edge was coated with plaster of Paris. Only the middle of the remaining stipe was touched with forceps. Fruit bodies at growing stage III (caps somewhat opened) (Hackman & Meinander 1979) were used for all tests.

Each species was tested separately using the same protocol. Four treatments were used. Collembola were provided with: (1) a fruit body of the test fungus with intact cystidia; (2) a fruit body of the test fungus with the cystidia destroyed (see below); (3) edible food (interior tissues of *P. nameko* fruit bodies as a positive control); or (4) no food (as a negative control). The first two treatments examined the effects of cystidia, whereas the latter two treatments examined the feeding status and background mortality of the tested collembola.

For the cystidium destruction treatment, cystidia were destroyed or removed by wiping the fruit body with filter paper immediately before use. Destruction or removal of cystidia could be recognised by changes in texture: dense cystidia resulted in a velvety texture (Fig 1), whereas the destroyed area had a smooth texture. For *R. bella*, pleurocystidia were not destroyed because few animals were found dead on the sides of gills in the field (see Results) and pleurocystidia were very sparse.

Ten collembola were transferred into each test vessel (30 mm diam, 50 mm height) with moist plaster at the bottom (5 mm depth) with a single test fruit body, edible food, or no food. The vessels were covered with nylon mesh (50-μm mesh). All vessels were placed in the dark at 23 °C or 20 °C for *R. bella* or *S. ohshimae*, respectively, in a larger vessel with water at the bottom (3 mm deep) and sealed tightly. After 48 h, the mortality of collembola and their location in the vessels were determined. The absence of activity, even when tapped with a pig hair, was regarded as indicative of death. There were three replicates per treatment.

Laboratory observations of collembolan death on cystidia

The behaviour of collembola on the dense cheilocystidia of *Russula bella* and on cystidia of *Strobilurus ohshimae* gills was observed in the laboratory until death. The combinations of test species were the same as in the cystidium-destruction experiments. Collembolan specimens for the *R. bella* and *S. ohshimae* tests were collected from Minamisaku, maintained at 23 °C and fed *Pholiota nameko* on culture substrates until use. Fruit bodies of *R. bella* were collected from Minamisaku 1 d before testing and stored at 8 °C until use. Fruit bodies of *S. ohshimae* were collected from laboratory cultures. Fruit bodies at growing stage II (gills folded; Hackman & Meinander 1979) were used.

A fruit body with intact cystidia was placed upside down in a chamber lined with moist filter paper. One collembolan was transferred onto the fungus using a pipette and the time until death (i.e. absence of activity, even when tapped with a pig

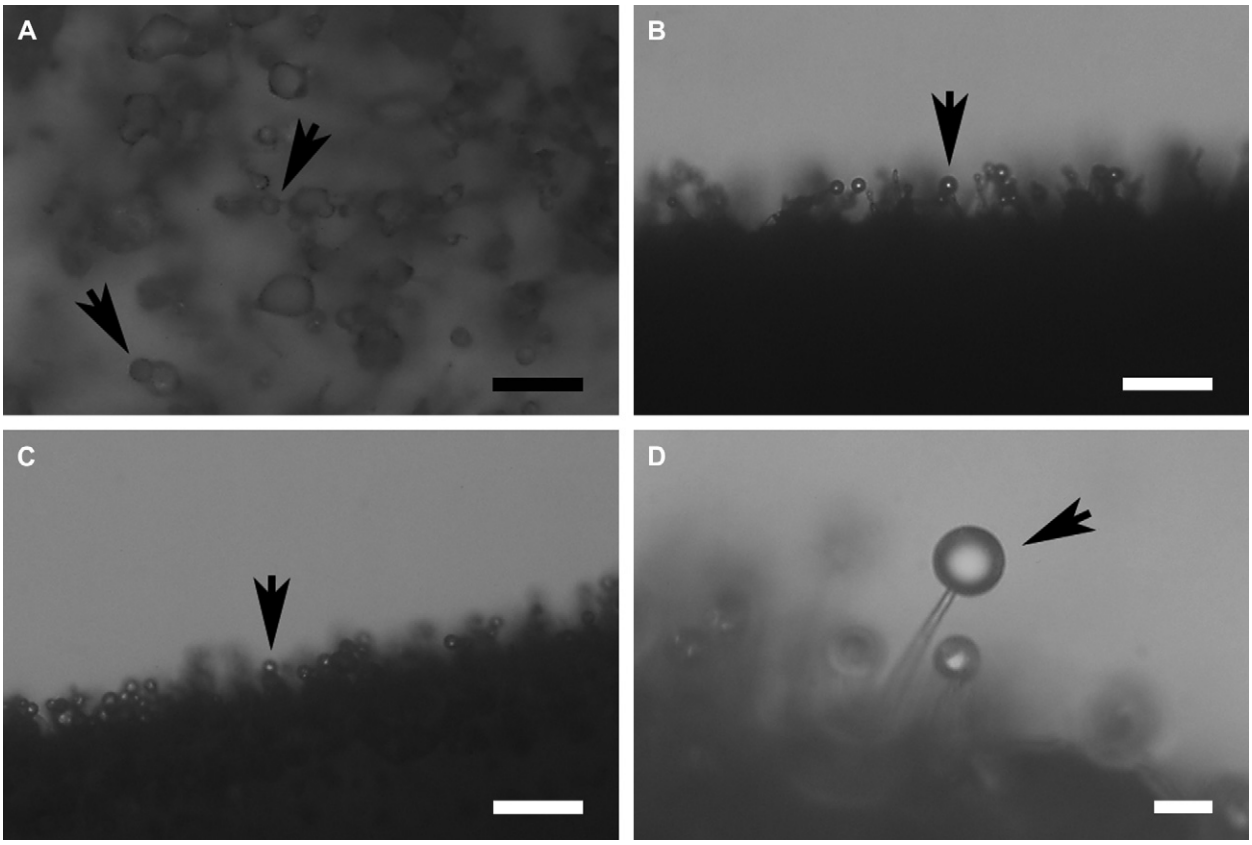

Fig 1 – *Russula bella* cystidia on the cap (A), stipes (B, D) and the edge of a gill (C). Arrow indicates a droplet secreted by an intact cystidium. Bars = (A–C) 50 μm, (D) 10 μm.

hair) was recorded. Observations under a binocular microscope were performed continuously from the initial collembola placement for 30 min, then at 1-min intervals to 1 h, and then at 10-min intervals until death. The experiment was conducted at 23 °C in the light. There were nine replicates with *Ceratophysella denisana* and 14 with *Mitchellania horrida* on *R. bella* (using a total of 11 fruit bodies), and five with *C. denisana* on *S. ohshimae* (using five fruit bodies). As a control, the same number of individuals was transferred to moist filter paper instead of to a fungus, and mortality was examined after 60 min for the test on *R. bella* and after 24 h for the test on *S. ohshimae*. The experimental period was longer than the survival time of collembola on the fungi.

Sensitivity of basidiospores to feeding by collembola

The sensitivity of *Russula bella* basidiospores to feeding by *Ceratophysella denisana* and *Mitchellania horrida*, and of *Strobilurus ohshimae* basidiospores to feeding by *C. denisana* was assessed. *C. denisana* and *M. horrida* were collected from Otsu and Chiba, respectively, and were fed *Pholiata nameko* on culture substrates. Fruit bodies of *R. bella* and *S. ohshimae* were collected from Minamisaku and Chiba, respectively.

Paired comparison of intact and gut-passed basidospores, obtained from a single fruit body, were made. The methods followed those of Nakamori & Suzuki (2005b) with some modifications. Briefly, basidiospores were collected from a fruit body on discs of black paper and fed to a collembolan. Faeces defecated by the individual were mounted on a glass slide in an aqueous solution of 1 % phloxine B. A total of 200 basidiospores in the faeces of each individual were examined microscopically. As a control, 200 intact basidiospores from another disc from the same fruit body were examined. Basidiospores were considered broken if the cell contents were lost; all others were considered unbroken. There were 12 replicates for each combination of fungal and collembolan species. Observations were carried out at 20 °C in the light.

Because the faecal pellets of *C. denisana* eating *S. ohshimae* were too soft to pick up, a collembolan individual together with a drop of water was transferred into a space made with Parafilm between a glass slide and coverslip for defecation. Faeces that were defecated on the glass slide or coverslip were mounted. Because basidiospores of *S. ohshimae* in the faeces were highly fragmented and uncountable, fragments that appeared to be from more than 200 basidiospores were examined.

Identification and preservation of specimens

To avoid killing collembola and damaging fruit bodies before the experiments, specimens were identified macroscopically and identification was confirmed based on microscopic morphology at the end of the experiments. Reference specimens are preserved in the Natural History Museum and Institute, Chiba: *Russula bella* (CBM-FB-36765), *R. violeipes* (CBM-FB-36766), *Strobilurus ohshimae* (CBM-FB-36978), *Ceratophysella*

denisana (CBM-ZI-133148 and 133149 for specimens from Minamisaku and Otsu, respectively), and *Mitchellania horrida* (CBM-ZI-133150 and 133151 for specimens from Minamisaku and Chiba, respectively).

Statistical analyses

In the cystidium-destruction experiment, data were pooled within treatments, and differences in mortality were analysed for all pair-wise comparisons using a Fisher's exact test with the Holm correction. Differences in the location of collembolan survivors were analysed using Fisher's exact test with the data pooled within treatments. In the laboratory observations of collembolan death on cystidia, differences in mortality between control and cystidium treatments were tested with a Fisher's exact test. Species differences in survival time were analysed using a Cox-regression (Cox 1972). The percentage of broken spores in faeces was compared with a Wilcoxon signed rank test.

Results

Microscopic observations of intact Russula bella *cystidia*

Droplets were observed on the pileo-, caulo-, cheilo- and pleurocystidia of *Russula bella* (Fig 1), and these secretions adhered to glass when touched. The secretions seemed to be insoluble or poorly soluble in water.

Location of dead collembola on Russula bella *fruit bodies in the field*

The field-collected *Russula bella* fruit bodies ($n = 16$) had several species of dead collembola stuck to their surfaces (Fig 2A–E). The collembolan species were *Ceratophysella denisana*, *C. denticulata*, *Desoria trispinata, Entomobrya* spp., *Mitchellania horrida*, *Sphaeridia pumilis,* and an undetermined species of *Symphypleona*. Most were dead on fruit body parts with abundant cystidia (i.e. caps, stipes, and edges of gills) and most seemed to be held in place by the cystidia. Only 3 % ($n = 72$) of dead individuals were found on parts of fruit bodies with sparse cystidia (i.e. the sides of gills). Additionally, one dead fly was found on a fruit body (Fig 2F).

Dead collembola (*Folsomia octoculata*, *Hypogastrura* sp., *M. horrida*, *Pseudachoratus* sp., and *Superodontella* sp.) were also found on field-collected fruit bodies of *R. violeipes* ($n = 3$). Most individuals were found on parts with abundant cystidia (89 %, $n = 19$) and seemed to be held in place by the cystidia.

Cystidium-destruction experiment on Russula bella *and* Strobilurus ohshimae

No individuals died in the edible-food and no-food treatments (Fig 3), and all individuals in the edible-food treatment showed signs of feeding. Therefore, the tests had base values of 0 % mortality and 100 % feeding.

In the experiments on *Russula bella*, the mortality was significantly higher in the intact-cystidium treatment than in the destroyed-cystidium treatment, the edible-food (positive control) treatment and the no-food (negative control) treatment for both *Ceratophysella denisana* and *Mitchellania horrida* (Fig 3; Fisher's test with Holm correction at $P < 0.05$, data from all replicates). Mortality on *R. bella* with destroyed cystidia was not significantly higher than in the two controls, although a few *M. horrida* individuals died (Fig 3; Fisher's exact test with Holm correction at $P < 0.05$, data from all replicates). Dead individuals were located mainly on the edges of gills and on the stipes, where cystidia were abundant. Survivors were found on the plaster substrate and on the parts of fruit bodies where cystidia were sparse or absent (i.e., opening between gills and the interior of fruit bodies). Survivors found on *R. bella* ate the fruit bodies.

In the experiment on *Strobilurus ohshimae*, the mortality of *C. denisana* in the intact-cystidium treatment was higher than in the other treatments, but not significantly so (Fig 3; Fisher's exact test, chi-square = 0.5, $P = 0.49$ for each comparison, data from all replicates). Dead individuals were located on the edges of caps. Survivors were found on the plaster substrate or on the cystidium-destroyed gills. Survivors found on *S. ohshimae* ate the fruit bodies.

The number of collembolan survivors on gills was significantly higher in the destroyed-cystidium treatment than in the intact-cystidium treatment for *M. horrida* on *R. bella* and for *C. denisana* on *S. ohshimae* [47 % ($n = 30$) *versus* 13 % ($n = 30$) and 23 % ($n = 30$) *versus* 0 % ($n = 30$), Fisher's exact test, chi-square = 6.4 and 7.1, $P = 0.010$ and 0.004, respectively, data from all replicates], but not for *C. denisana* on *R. bella* [36 % ($n = 30$) *versus* 27 % ($n = 30$), Fisher's exact test, chi-square = 0.3, $P = 0.6$, data from all replicates].

The number of survivors not on fruit bodies was significantly higher in the intact-cystidium treatments than in the positive control [*C. denisana* on *R. bella*: 40 % ($n = 30$) *versus* 0 % ($n = 30$), *M. horrida* on *R. bella*: 43 % ($n = 30$) *versus* 0 % ($n = 30$), and *C. denisana* on *S. ohshimae*: 93 % ($n = 30$) *versus* 0 % ($n = 30$); Fisher's exact test, chi-square = 12.6, 14.1, and 48.8, respectively, all $P < 0.001$, data from all replicates]. However, the number did not differ significantly between the intact-cystidium and destroyed-cystidium treatments [*C. denisana* on *R. bella*: 40 % ($n = 30$) *versus* 63 % ($n = 30$), *M. horrida* on *R. bella*: 43 % ($n = 30$) *versus* 47 % ($n = 30$), and *C. denisana* on *S. ohshimae*: 93 % ($n = 30$) *versus* 73 % ($n = 30$); Fisher's exact test, chi-square = 2.4, 0, and 3, $P = 0.120$, 1, and 0.080, respectively, data from all replicates].

Laboratory observations of collembolan death on cystidia

No individuals died on the moist filter paper, whereas all individuals died on cystidium-bearing fruit bodies (Fisher's test, chi-square = 14.2, 24.1, and 6.4, $P < 0.001$, for *Ceratophysella denisana* on *Russula bella*, *Mitchellania horrida* on *R. bella*, and *C. denisana* on *Strobilurus ohshimae*, respectively).

The median (range) survival time for *C. denisana* on *R. bella*, *M. horrida* on *R. bella*, and *C. denisana* on *S. ohshimae* was 724 s (288–930 s; $n = 9$), 36 (2–79 s; $n = 14$), and 4620 s (4020–10800 s; $n = 5$), respectively. Survival time on *R. bella* differed between collembolan species: *M. horrida* died significantly faster than *C. denisana* (Cox-regression, $z = 5.4$, $P < 0.001$). When individuals were on the dense cystidia of *R. bella*, their movement

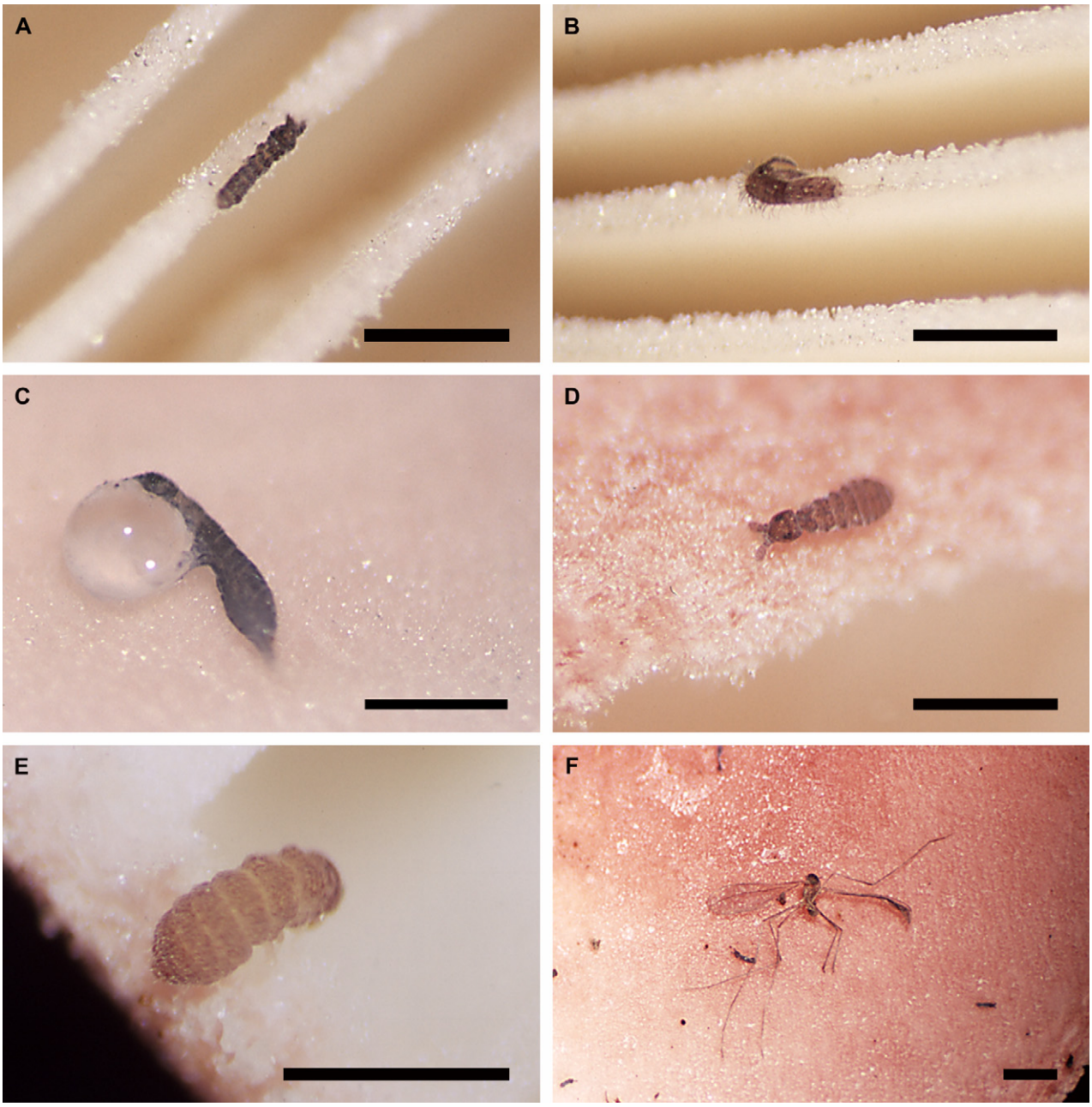

Fig 2 – Collembola (A–E) and a fly (F) found dead on *Russula bella* fruit bodies in the field. (A) *Ceratophysella denticulata* on the edge of a gill, (B) *Entomobrya* sp. on the edge of a gill, (C) *Desoria trispinata* on a stipe, (D–E) *Mitchellania horrida* on caps, (F) fly on a cap. Bars = (A–E) 0.5 mm, (F) 1 mm.

was arrested quickly, especially for *M. horrida*. Cystidial secretions stuck to the body surfaces, including the mouth and anal regions, legs, furcae, and antennae. No individuals showed signs of feeding.

The survival time of *C. denisana* on fruit bodies differed between fungal species: *C. denisana* died significantly faster on *R. bella* than on *S. ohshimae* (Cox-regression, $z = 4.2$, $P < 0.001$). On *S. ohshimae*, cystidial secretions were also present and found stuck to collembolan body surfaces. No individuals showed signs of feeding.

Sensitivity of basidiospores to feeding by collembola

The percentage of broken spores was significantly higher in collembolan faeces than in uneaten controls for all tests: medians (ranges) were 100 % (100 %), 98.5 % (88–100 %), and 100 % (100 %) for *Russula bella* in *Ceratophysella denisana* faeces, *R. bella* in *Mitchellania horrida* faeces and *Strobilurus ohshimae* in *C. denisana* faeces, respectively, *versus* 0 % (0 %) for all uneaten controls (Wilcoxon signed rank test, $P < 0.001$, $n = 12$ each). No collembola died.

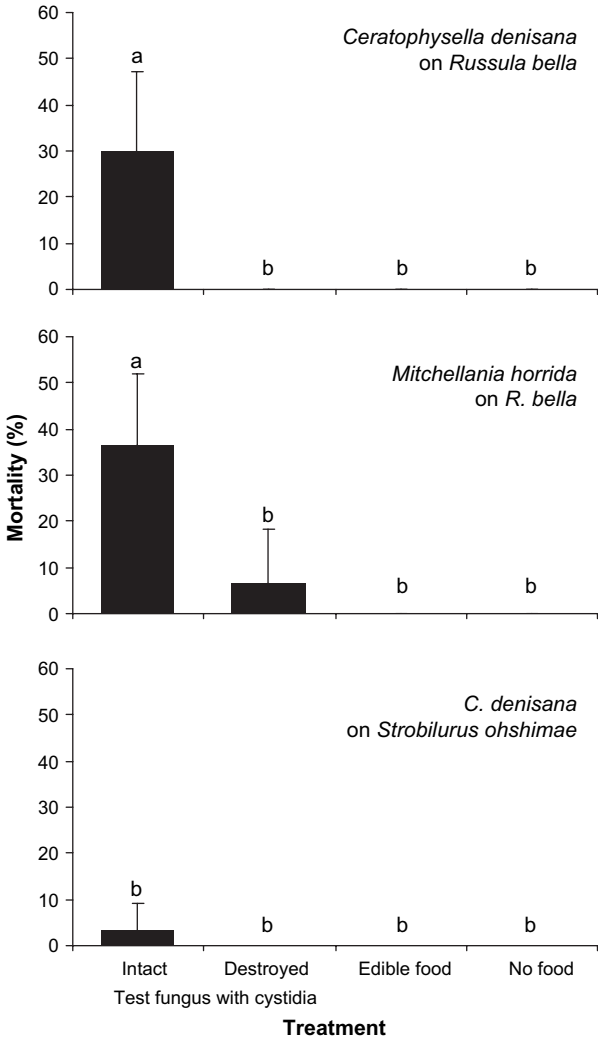

Fig 3 – Mortality of collembola in the cystidium-destruction experiment. Error bars indicate standard deviations ($n = 3$); different letters above bars indicate significant differences in mortality rates (multiple comparisons by Fisher's exact test with Holm correction at $P < 0.05$, accumulated data of replicates).

Discussion

Our results showed that collembola can extensively damage *Russula bella* and *Strobilurus ohshimae* basidiospores by feeding on them. Additionally, collembola can aggregate on a single fruit body by the thousands (Sawahata *et al.* 2000), and feed on basidiospores or on tissues in which basidiospores are produced (Sawahata 2006; Sawahata *et al.* 2000). The number of live collembola on gills indicates the intensity of basidiospore grazing.

The cystidia of *R. bella* and *S. ohshimae* may protect basidiospores from collembola or other small fungivores. The cystidium-destruction experiment revealed that cystidia reduced the number of live collembola on gills, where they can damage basidiospores, suggesting a defensive role of cystidia, at least for the species studied, although the effects were not significant for *R. bella* against *Ceratophysella denisana*. Furthermore, cystidia of *R. bella* increased collembolan mortality in the laboratory; in the field, collembola were found dead on the parts of fruit bodies where cystidia were abundant, suggesting that the deadly cystidia may be selected in *R. bella* to reduce collembolan attacks on the fruit bodies. Indeed, approximately one-third of the collembola, even those in the feeding phase, appeared to avoid *R. bella* in the cystidium-destruction experiment. Thus, the presence of cystidia may protect basidiospores by reducing basidiospore damage due to collembolan ingestion. Additionally, the cystidia of *R. bella* may adversely affect other fungivores, as evidenced by the dead fly being found on an *R. bella* fruit body.

The number of individuals that did not attack fruit bodies was not affected by cystidium destruction, although cystidia are thought to cause collembolan avoidance of fruit bodies. Our explanation of this finding is that collembola may avoid cystidium bearing fungi using cues other than the cystidia themselves. Similarly, some collembola can detect toxic fungi using cues other than the toxin (Scheu & Simmerling 2004).

Our results suggest that *R. bella* cystidia are capable of killing *C. denisana* and *Mitchellania horrida* on contact. Cystidial secretions may cause collembolan death because the secretions stick to collembola when they come into contact with cystidia. The death of *M. horrida* in the destroyed-cystidium treatment may be explained by adhesion to secretions remaining on the fruit bodies. The pleurocystidia of *R. bella* may not be lethal or may be too sparse to kill collembola because the undestroyed pleurocystidia did not result in significant mortality. Although the oral toxicity of cystidia remains unclear, areas of the fruit bodies that had no or sparse cystidia were eaten by collembola that survived in the laboratory. Thus, ingestion of fungal tissues may not adversely affect collembola.

Collembola-killing cystidia are likely to occur in other fungi. Cystidia of *S. ohshimae* may be lethal to collembola because collembola died on the cystidium-bearing surfaces of fruit bodies in the laboratory. The lack of significant effects of cystidia on collembolan mortality in the cystidium-destruction experiment may be because *S. ohshimae* cystidia are less lethal. The possible lethal effects may be involved in the collembolan avoidance of *S. ohshimae* observed in the cystidium-destruction experiment. Cystidia of *R. violeipes* may also be lethal because the fruit bodies have cystidia similar to those of *R. bella* and were also found with dead collembola. Additionally, cystidia with calcium oxalate crystals on the apices may be harmful to collembola because calcium oxalate is a known toxin or physical barrier in some plants (Franceschi & Nakata 2005). However, the effects of these cystidia on collembola remain to be examined.

The cystidia of other species may have functions other than affecting or killing collembola, e.g. gill spacing or excretion (Largent *et al.* 1977). Because there are interspecific differences in the size, form, chemical composition, abundance, location, and origin of cystidia (Largent *et al.* 1977), their function may be species-specific. Live collembola have been collected from cystidium-bearing fungal species of *Boletaceae*, *Cortinariaceae*, and *Russulaceae* (Castaño-Meneses *et al.* 2004; Cave 1997; Mateos *et al.* 1996; Nakamori & Suzuki 2005a; Palacios-Vargas & Gómez 1991; Sawahata *et al.* 2000), although these studies did not focus on dead collembola.

In conclusion, the protection of basidiospores from collembolan grazing is one possible function of cystidia. The dense cystidia of *R. bella* and *S. ohshimae* are capable of affecting or killing collembolan species that are potential basidiospore predators. This supports the hypothesis that cystidia may protect fruit bodies from predators (Buller 1909), at least for *R. bella* and *S. ohshimae*. Because cystidia are found on the fruit bodies and other structures of many species, our findings shed new light on fungus–collembolan interactions.

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