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# *Rickia wasmannii* increases the need for water in *Myrmica scabrinodis* (Ascomycota: Laboulbeniales; Hymenoptera: Formicidae)

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## ABSTRACT

The order Laboulbeniales (Fungi, Ascomycota) is a little-studied group of microscopic ectoparasites of invertebrates, mostly insects. The effects of Laboulbeniales species on their hosts are mostly unknown. *Rickia wasmannii* Cavara, 1899 is a common Laboulbeniales fungus occurring in Europe and is currently known to be a parasite of at least eight *Myrmica* ant species. *R. wasmannii* serves as a good model organism for Laboulbeniales–host interactions, as this species covers the host in a very high density, and infected host individuals can be easily collected in high numbers. The effect of *R. wasmannii* on the survival rate of its most common host species, *Myrmica scabrinodis* Nylander 1846, was therefore investigated in a laboratory experiment on an individual level. To enhance the results, environmental stresses were simulated by depriving infected and uninfected workers of water and food. The survival of individuals was recorded hourly until the death of the last individual. Infected specimens were significantly more sensitive to the withdrawal of food and water than uninfected specimens. When we tested for water consumption, we found that infected ants spent more time consuming water than uninfected ants. Therefore, it is possible that infected ants must replace the loss of water. Based on these results, *R. wasmannii* substantially decreases the chances and time of survival of infected individuals, at least in resource-limited environments, which suggest that *R. wasmannii* has a negative effect on its host.

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## 1. Introduction

The Laboulbeniales are an ascomycetous order of fungi comprising 140 genera and more than 2000 species (Rossi and Santamaria 2012). They are obligate ectoparasites of arthropods, especially insects. Laboulbeniales fungi parasitize at least twelve different insect orders but most commonly (about 90% of species) they parasitize Coleoptera and Diptera (Henk et al., 2003; Santamaria, 2001). They are basically thought to be spread via direct contact between individual hosts (e.g., Cottrell and Riddick, 2012; De Kesel, 1996). Most Laboulbeniales fungi exhibit extreme host-specificity; the host spectrum ranges from single (stenotopic) to multiple host species (eurytopic) (Haelewaters et al., 2012 and references therein). In contrast with most fungus species, only the sexual stages of Laboulbeniales have been found (Haelewaters, 2012). Little is known about the effect of Laboulbeniales fungi on their host species. Espadaler and Santamaria (2012) mention their “ability to grow on their hosts without inflicting any noticeable injury”. They are sometimes

considered to be a neutral factor (Garcia et al., 2010; Lapeva-Gjonova and Santamaria, 2011; Whisler, 1968), or to cause little to no harm (Benjamin, 1971; Majewski, 1994; Santamaria, 1998; Scheloske, 1969). Negative effects are also mentioned (Benjamin, 1971; Nalepa and Weir, 2007; Raak-van den Berg et al., 2014; Riddick, 2010, 2006). Gemeno et al. (2004) found some negative effects of a Laboulbeniales fungus on a cockroach and Strandberg and Tucker (1974) in an earwig species. However, the infection rate on the hosts and the number of infected individuals are usually too small to study such questions in wild populations.

Ants are the only known hosts of Laboulbeniales in the order Hymenoptera (Espadaler and Santamaria, 2003). Four species of Laboulbeniales fungi have been reported so far to parasitize ants in Europe: *Rickia wasmannii* Cavara (Fig. 1) in 15 countries; *Laboulbenia formicarium* Thaxt. in France, Portugal, and Spain; *Laboulbenia camponoti* S.W.T. Batra in Austria, Bulgaria, Hungary, and Spain; and *Rickia lenoirii* Santam. in Greece and France (Espadaler and Santamaria, 2012; Haelewaters, 2012; Báthori et al., 2014; Santamaria and Espadaler, 2015). *R. wasmannii* was understudied for a long time, but in recent years more reports on its distribution and host ant usage (for a review about both topics: Espadaler and Santamaria, 2012) have been published, and these have shown *R.*

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87 *wasmannii* to be the most widely distributed of the four ant-parasitizing Laboulbeniales species in Europe. Reports from England, 88 France, Germany, Luxembourg, the Netherlands, Switzerland, 89 Poland, Italy, Austria, Slovenia, the Czech Republic, Slovakia, Hun- 90 gary, Romania and Bulgaria have shown *R. wasmannii* populations 91 parasitizing eight species of the *Myrmica* Latreille 1804 (Hymenop- 92 tera: Formicidae) genus: *Myrmica slovacica* Sadii, 1952; *M. scabrinodis* 93 Nylander, 1846 (Fig. 1); *M. specioides* Bondroit, 1918; *M. vandeli* 94 Bondroit, 1920; *M. rubra* (Linnaeus, 1758); *M. sabuleti* Meinert, 95 1861; *M. gallienii* Bondroit, 1920 and *M. ruginodis* Nylander, 1846 96 (Espadaler and Santamaria, 2012; Haelewaters, 2012; Csata et al. 97 2013; Santamaria and Espadaler, 2015). In Hungary, it is the only 98 ant-parasitizing Laboulbeniales species found (Tartally et al., 2007). 99

100 Despite the increasing number of papers describing the distribu- 101 tion of *R. wasmannii* (Csata et al., 2013; Espadaler and Santamaria, 102 2012, and references herein; Haelewaters, 2012), there is little infor- 103 mation about the interactions between this fungus and its hosts. As 104 *R. wasmannii* is known to be the most widely distributed ant-parasitizing 105 Laboulbeniales species in Europe (see above), and as highly- 106 infected *M. scabrinodis* (the commonest host in the Carpathian 107 Basin: Csata et al., 2013; Tartally, 2008; Tartally et al., 2007) workers 108 with this fungus can be easily collected in high numbers (pers. 109 observ.), we surmised that *R. wasmannii* would be a good model 110 organism of the Laboulbeniales order to study the interactions 111 between Laboulbeniales fungi and their hosts, collected from wild 112 (rather than laboratory, as in, e.g., Cottrell and Riddick, 2012; 113 Gemeno et al., 2004) populations. One of the most important ques- 114 tions about such interactions is whether Laboulbeniales fungi have 115 any negative or positive effects on the host organism. A recent paper 116 has already demonstrated negative effects of the fungi on its *M. scab-* 117 *rinodis* host: workers infected with *R. wasmannii*, have a reduced life- 118 span, while they display increased allo-grooming behaviour (Csata 119 et al., 2014). In this research, environmental stresses were simulated 120 by withdrawing water and food from four groups of *M. scabrinodis* 121 workers, two infected by *R. wasmannii* and two uninfected. The sur- 122 vival rate of these four groups without food and water, and their 123 water consumption after water-deprivation were compared.

124 **2. Materials and methods**

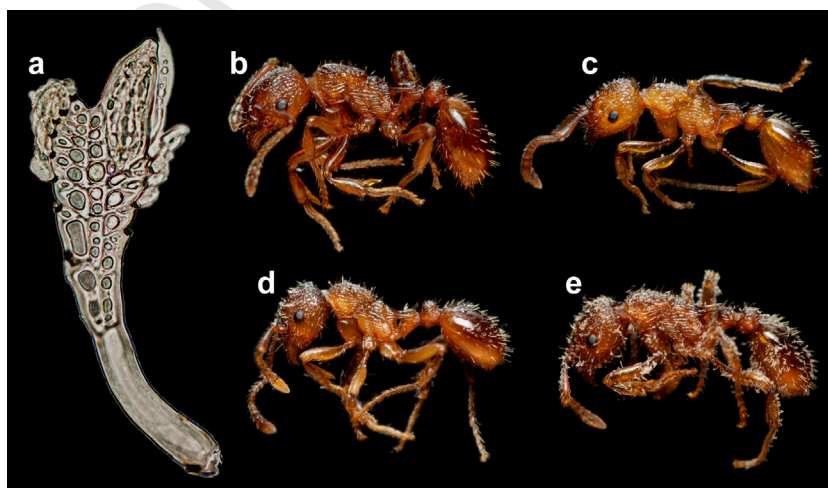
125 **2.1. Ant colony collection**

126 Despite the intensive work of one of the authors (AT) on sites 127 with *R. wasmannii* (see e.g. Tartally, 2008 and references therein),

128 no single sites with highly infected and uninfected *M. scabrinodis* 129 colonies were found in proportions that would have been useful 130 in collecting infected and uninfected (control) colonies. We found 131 that the known sites in Hungary contain almost exclusively 132 highly-infected or uninfected colonies of host ant species. Accord- 133 ingly, *M. scabrinodis* colonies were collected from marshy mead- 134 ows of two different regions in Hungary in order to make 135 comparisons in two cases. Twelve colonies were collected from 136 northern Hungary (six infected colonies from Meszes: 48°27'N, 137 20°47'E, 165 m a.s.l. and six uninfected colonies from Aggtelek: 138 48°26' N, 20°30'E, 340 m a.s.l.) and twelve others from eastern 139 Hungary (six infected colonies from Újléta: 47°26'N, 21°51'E, 140 120 m a.s.l. and six uninfected ones from Csíkgát at Monostorpályi: 141 47°25'N, 21°48'E, 108 m a.s.l.). All 24 colonies contained fertilized 142 queens, hundreds of workers, larvae, and pupae. The ants were 143 kept in artificial lab nests at 23 ± 1 °C with complex food resources 144 (cockroaches twice a week and honey water ad libitum). Plastic 145 boxes were used as formicaria (length: 16.5 cm, width: 11.5 cm, 146 height: 6 cm; painted with Fluon® in the inner walls to prevent 147 the ants from escaping). The box bottoms were covered with plas- 148 ter, and they all contained a chamber (length: 5.5 cm, width: 149 4.5 cm, height: 1 cm) covered by glass plates. All colonies were 150 stored in the laboratory for a minimum of 1 month before the 151 experiment as an acclimatisation period.

152 **2.2. Experimental design**

153 Five days before the experiment, 20 workers were randomly 154 selected from each of the 24 colonies and moved together to open 155 plastic boxes (12 \* 20 infected and 12 \* 20 uninfected workers were 156 selected = 480 workers in total). The same boxes were used as above 157 but without plaster. The boxes were uncovered to allow for evapo- 158 ration. In order to make the research repeatable, the separated mini- 159 colonies were provided with Bhatkar diet (Bhatkar and Whitcomb, 160 1970) and water ad libitum 24 h before monitoring. After 24 h, all 161 food and water were withdrawn, and each mini-colony was checked 162 hourly by counting the number of dead workers. A specimen was 163 considered to be dead when it did not move its legs or antennae 164 after they were touched with forceps. Dead individuals were 165 removed from the mini-colonies following the hourly counting. 166 During the experiment the room temperature (23.4–24.2 °C) and 167 humidity (35–39%) were recorded hourly. The experiment ended 168 with the death of the last individual (55 h after the beginning). By 169 the end of the experiment, the fungal thalli on the dead infected



170 **Fig. 1.** *Rickia wasmannii* thallus (a) and *Myrmica scabrinodis* workers uninfected (b) and infected (c–e) in three orders of magnitude (c: 9, d: 98, e: 986 thalli; photos by Walter P. Pfliegler).

individuals were counted under a Leica MZ12.5 stereomicroscope at magnifications of 10×–160×.

To test whether the limiting factor of the survival of the ants was the deprivation of water only or also the deprivation of food, a control experiment was carried out in which food was withheld but water was provided ad libitum. This experiment involved a total of 480 specimens, 20 individuals from each of the 12 infected and 12 uninfected colonies described above. This experiment ended 55 h (see the end of the previous paragraph) after food deprivation, when the number of dead individuals was counted in each nest. The opposite control experiment was not possible because of the water content of the food.

In the third part of the research, the water consumption of infected and uninfected *M. scabrinodis* workers was tested under laboratory conditions. We measured the time that one individual spent consuming water after a minimum of 12 h of water deprivation using the same 24 ant colonies described above. For this experiment, 20 individuals were selected from each nest to form mini-colonies. After 12 h of water deprivation, 240 uninfected and 213 infected workers survived, and they were all moved separately and randomly to different test tubes (length = 53 mm, *d* = 15 mm). After a one-minute acclimatisation period 0.05 ml of water was introduced into the test tubes and the ants were given three minutes to discover it. The individuals that did not discover the water within this three-minute period were excluded from the test (16 infected and 11 uninfected). Thus, we worked practically with 197 infected and 229 uninfected *M. scabrinodis* workers. The length of time a worker spent in the first round of water consumption was noted in the case of all 426 individuals.

### 2.3. Statistical analyses

Statistical analyses were carried out using R statistical software (R Development Core Team R, 2011). To compare the survival curves Mixed Effect Cox regressions (Cox, 1972 as implemented in the coxme add-on package in R (Therneau, 2012)) were used. The Generalized Linear Mixed Model (GLMM, Poisson error, maximum likelihood fit) approach was applied to test the effects of infection on the water consumption of individuals. The length of time an individual was deprived of water before the water consumption experiments was introduced as a covariate. The interaction between the length of this period of time and the infection status of the observed individuals was also taken as a covariate. For the survival rates of infected and uninfected ants, in both cases (Cox regression and GLMMs) colony identity was included as a random factor, and the infection status of the ants (infected vs. uninfected), habitat type (Újléta, Meszes, Csíkgát, Aggtelek) and categories of fungal thalli were included as a dummy variable. GLMMs were performed using glmer function in the lme4 package (Bates et al., 2014). In the case of survival of the infected ants, they were categorized according to the number of fungal thalli (see examples at: Fig. 1) on an individual level. Fifty categories were created for infected ants by scale 20 (“category 1”: 1–20 thalli, “category 2”: 21–40 thalli and so on up to “category 50”: 981–1000 thalli). No other categories were necessary because the highest recorded number of thalli was 986 per individual. Uninfected individuals were assigned to “category 0”. Bonferroni Holm correction was used to determine the levels of significance when Cox regression (population level) and GLMM (category level) analyses were performed.

### 3. Results

While the first infected worker died 5 h after the beginning of the experiment and the last 28 h later (33 h after the beginning of the experiment), the first uninfected worker died 12 h after

the start of the experiment and the last one 43 h later (55 h after the beginning of the experiment). When the infected workers (Újléta and Meszes) were compared as a group with the uninfected ones (Csíkgát and Aggtelek), the lifespan of the uninfected workers was found to be significantly higher (Cox regression, coeff = 1.45, exp (coef) = 4.30, *z* = 13.58, *p* < 0.0001, *n* = 468). When uninfected and infected workers were compared at the population level, similar results were found, since survival rates of the two infected populations, Meszes and Újléta, were significantly different from survival rates of the two uninfected populations, Csíkgát and Aggtelek (Fig. 2: Meszes: *z* = −6.63, *p* < 0.0001; Újléta: *z* = −4.81, *p* < 0.0001). However, no significant differences were found between the infected Újléta and Meszes populations (*z* = −0.28, *p* = 0.77). The same results were obtained in the case of uninfected populations, as there were no significant differences between the Csíkgát and Aggtelek populations (*z* = −1.19, *p* = 0.46). The number of fungus thalli had a significant effect on the survival rate of the infected individuals (*z* = −7.39, *p* < 0.0001). Thus, heavily parasitized individuals died significantly more rapidly than lightly parasitized individuals. As a comparison of the lifespans of uninfected and infected workers on the basis of the infection categories revealed, individuals belonging to the higher categories (“category 2–50”) of infection died significantly more rapidly than those from “category 0” (Fig. 3: GLMM: *z* = −8.25, *p* < 0.0001, *n* = 468). Infected individuals from “category 2” died significantly more rapidly (GLMM: *z* = −2.39, *p* = 0.03) than uninfected ones from “category 0”. In the second experiment, when only food was withheld, only 35 dead specimens were recorded among the 240 infected ants (14.58% mortality rate) and 4 among the 240 uninfected individuals (1.66% mortality rate).

In the case of water consumption, a significant difference was found between the populations from Aggtelek and Meszes, as infected individuals spent more time consuming water than uninfected ones (GLMM: *z* = 2.72, *p* = 0.006, *n* = 197). Similar differences were found between populations from Újléta and Csíkgát (GLMM: *z* = 3.91, *p* = 0.001, *n* = 229). By analysing all the infected (Újléta and Meszes) workers in comparison with all the uninfected workers (Csíkgát and Aggtelek), the results also showed that infected individuals spent significantly more time consuming water than uninfected ones (Fig. 4: GLMM, *z* = 5.4, *p* = 0.001).

### 4. Discussion

The results (Fig. 2 and 3) clearly show that *R. wasmannii* has a negative effect on the survival rate of *M. scabrinodis*, at least under

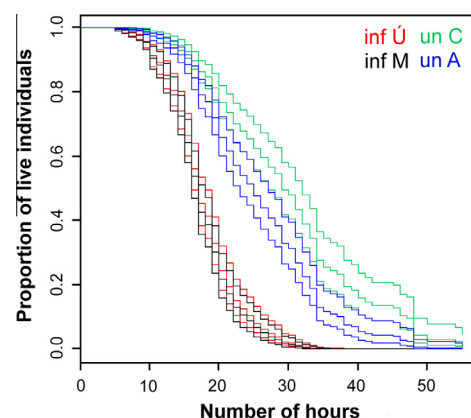


Fig. 2. The lifespan of infected (inf) and uninfected (un) *Myrmica scabrinodis* workers from four populations: Újléta (Ú), Meszes (M), Csíkgát (C) and Aggtelek (A). Broken lines represent a point-wise 95-percent confidence interval around the corresponding functions.



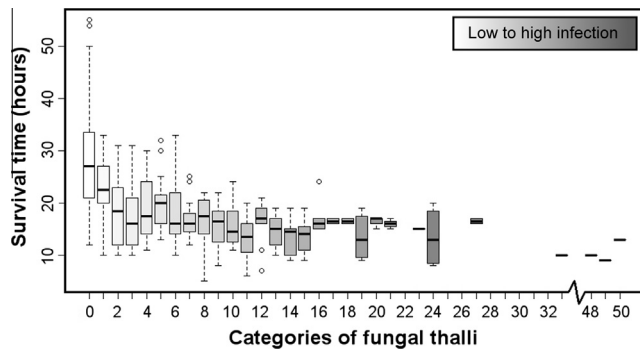


Fig. 3. Survivorship of all the analysed (see Fig. 2) *M. scabrinodis* workers ordered into 51 categories of infection (uninfected: “category 0”; infected: “category 1”: 1–20 thalli, “category 2”: 21–40 thalli and so on up to “category 50”: 981–1000 thalli).

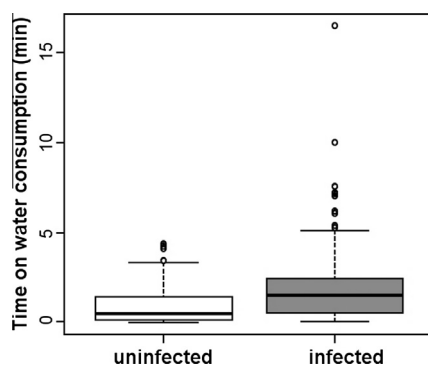


Fig. 4. Individual time (min) spent on water consumption in infected and uninfected populations.

laboratory conditions when water and food are not available. Similarly, with regards to water consumption the analysis showed that infected ants spent significantly more time consuming water than uninfected ants (Fig. 4).

We obtained these results from two different regions, but further similar research would be useful involving sites in which both infected and uninfected colonies are available in high numbers. It is not clear whether the effect of *R. wasmannii* on *M. scabrinodis* is so clearly negative under natural conditions, since, for example, the ants can dig down to wet soil in drier periods (Scherba, 1959). However, we can conclude that *R. wasmannii* has an influence on the physiology of *M. scabrinodis*. Further research is necessary to investigate the background of this influence. Possible options are (1) direct metabolic effects on the ants; (2) absorption of water from the hemolymph by fungal thalli; (3) the fungi create the conditions for intensified evaporation (as in the case of cockroaches: Gemeno et al., 2004); (4) the fungi place an additional burden on the ants, which increases the metabolic rates in the ants as they need extra energy in order to bear the additional weight; (5) the thalli on/around the mandibles prevent the ants from consuming water (see: Nalepa and Weir, 2007). Whatever the explanation may be, it seems clear that the ants must replace lost water, and this constitutes a negative effect of this fungus on the ant host. The results from *M. scabrinodis* individuals confirm the recent results of Csata et al. (2014), according to which *R. wasmannii* has a negative effect on *M. scabrinodis*.

As far as we know, these are among the first (see also Csata et al., 2014) results indicating the effects of an ant-parasitizing Laboulbeniales fungus on host ants. It would be useful to repeat such experiments on other ant-parasitizing Laboulbeniales species, such as *L. formicarum*. Ants infected by this fungus are also readily

available in high numbers (Herraz and Espadaler, 2007), but the other two European ant-parasitizing Laboulbeniales fungi are rather rare (Báthori et al., 2014; Espadaler and Santamaria, 2012; Santamaria and Espadaler, 2015) for the purposes of such research. Furthermore, it would also be useful to test for the negative effect of *R. wasmannii* not only on *M. scabrinodis* but also on other *Myrmica* species.

There are several ant-parasitizing fungi in different taxonomic groups (see e.g. Csata et al., 2013; Espadaler and Santamaria, 2012, and references therein), and based on our results *R. wasmannii* can also be defined as a parasite. However, it is not clear whether biological control of invasive ant species could be accomplished using *R. wasmannii* or other ant-parasitizing fungi, such as *L. formicarum*, which infects the invasive *Lasius neglectus* in Europe (see e.g.: Espadaler and Santamaria, 2012). Another example of insect-parasitizing Laboulbeniales fungi with potential as a biological control agent is *Hesperomyces virescens* Thaxt., which has a negative effect on the survival of the invasive *Harmonia axyridis* Pallas, 1773 ladybird males during wintering (Riddick, 2010; see: Raak-van den Berg et al., 2014 and its Supplementary material 1 for a review of negative effects of *H. virescens* on *H. axyridis*). In some cases, premature death or reduced fecundity has been observed (Bro Larsen, 1952; Kamburov et al., 1967a, 1967b). Further research on this question would also be useful in the case of invasive ants, but the potential positive results could be applied only after a strict control, because proper experiments should be done in order to determine if only the invasive, and not the native, ant species can be infected by any non-native pathogens.

The eight *Myrmica* species (see Section 1) currently known to be parasitized by *R. wasmannii* are widespread across Europe, although they are often found in small, isolated populations (Radchenko and Elmes, 2010). These host species are closely associated with other arthropods (see: Witek et al., 2014 for a review), for instance *Microdon* spp. (Diptera: Syrphidae), *Maculinea* spp. (Lepidoptera: Lycaenidae) and their ichneumon parasites (*Ichneumon* spp. and *Neotypus* spp.; Hymenoptera: Ichneumonidae), the latter often occurring where *R. wasmannii* are found (see: Appendix III of Tartally 2008). This implies that other arthropods might be potential vectors of *R. wasmannii* as long as they share the same habitat (sensu De Kesel and Haelewaters, 2014; Santamaria and Espadaler, 2015), but this is still an open question. Morphological and ecological evidence for host shift of Laboulbeniales species between unrelated but cohabiting host species was recently provided in De Kesel and Haelewaters (2014). Furthermore, these *Myrmica*-associated arthropods are often endangered (e.g. Munguira and Martín, 1999); therefore, a better understanding of the ecology of the “fungus-ant-social parasite system” is of crucial importance from a conservation biological point of view. Our results show that ants infected with *R. wasmannii* were less resistant to the absence of food and water (Figs. 2 and 3). Thus it is highly plausible that infected colonies have serious disadvantages against competitors and social parasites in their natural environments, a fascinating topic for future investigation.

According to Fig. 3, the heavier the parasitizing, the lower the survival rate. This finding confirms Crofton (1971a,b), who proposed that the death of hosts results only when hosts are heavily parasitized, when the reproductive potential of parasites exceeds that of the hosts, and when their frequency distribution is over-dispersed within host populations (i.e. when variance of fungal populations is significantly greater than the mean). This is because parasites (macroparasites in particular) tend to concentrate on a minority of heavily infested individuals (Clayton et al. 2003). By combining aspects of death and the dispersion of populations, Crofton (1971a,b) quantified and distinguished parasites from pathogens. According to these findings, further studies would be necessary on other Laboulbeniales species to conclude whether

they adopt a life history strategy similar to those of other parasites, with the potential for pathogenicity only when numbers are high within a given host individual.

The findings of earlier studies (e.g. Csata et al., 2014; Gemeno et al., 2004; Riddick, 2010; Strandberg and Tucker, 1974), according to which Laboulbeniales fungi can have a negative effect on the survival rates of their hosts, are confirmed (Figs. 2–4). We therefore hope that the recent results showing the negative effect of *R. wasmannii* on the survival rates of *M. scabrinodis* will be of interest from myrmecological, mycological, and parasitological viewpoints.

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