

Studies of Laboulbeniales (Fungi, Ascomycota) on *Myrmica* ants (II): variation of infection by *Rickia wasmannii* over habitats and time

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Abstract

One group of important insect parasites are the Laboulbeniales (Ascomycota), microscopic fungi that live attached to the exterior of their hosts, mainly beetles, but also mites, millipedes, earwigs, and ants. *Rickia wasmannii* is a common fungus in Europe and is limited to the ant genus *Myrmica* (Hymenoptera, Formicidae). This paper presents patterns of *R. wasmannii* infection in the Netherlands from three host species collected in three series of pitfall traps: *Myrmica ruginodis*, *M. sabuleti*, and *M. scabrinodis*. The infection rate of especially *M. sabuleti* was so high, that it allowed analyses of infection patterns over time and habitats. We found that only workers were infected, mostly the older ones with a hard cuticle. Gynes are probably never infected. This is supported with data from a nature restoration site: in this young area *R. wasmannii* is not abundant in contrast to close-by sites, so there probably is a build-up of infection by *Rickia* over time through worker contact. Taken over three periods throughout the year (*spring, summer, autumn*), parasite prevalence declined significantly in *M. sabuleti*, with a non-significant declining trend in *M. scabrinodis*. Increased allogrooming behavior in the nest in the winter may be the main contributing factor for this. New, largely uninfected cohorts of workers lead to decreased infection rate during the reproduction season. Finally, *Rickia wasmannii* occurs throughout a wide variety of habitats, from moist and cool to dry and warm.

Keywords

Auto- and allogrooming; ant-associated fungi; ectoparasites; Formicidae; Hymenoptera; Laboulbeniomycetes; parasite prevalence; variation of infection

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Introduction

The order Laboulbeniales (Ascomycota: Laboulbeniomycetes) consists of microscopic ectoparasites of Arthropoda, mostly true insects. Within the subphylum Hexapoda, representatives of nine orders are known as hosts: Blattodea, Coleoptera, Dermaptera, Diptera, Hemiptera, Hymenoptera, Mallophaga, Orthoptera, and Thysanoptera. [Note that the termites, previously ranked in the order Isoptera, were recently included in the order Blattodea, based on phylogenetic data (Beccaloni & Eggleton, 2013).] In addition, Acari (subphylum Cheliceriformes) and Diplopoda (subphylum Myriapoda) are known to host Laboulbeniales (Weir & Hammond, 1997; Haelewaters et al., 2012). Although being very diversified over different host orders, classes, and even subphyla, the majority of described species of Laboulbeniales parasitizes beetles (about 80%; Weir & Blackwell, 2005).

In the order Hymenoptera, only ants (family Formicidae) serve as hosts. Six species of Laboulbeniales have been described on ants: *Dimorphomyces formicicola* (Speg.) I.I. Tav. on *Nylanderia* [as *Paratrechina*] in Argentina; *Laboulbenia camponoti* S.W.T. Batra on *Camponotus* in Austria, Bulgaria, Romania, Spain, Turkey, and India (type); *Laboulbenia ecitonis* G. Blum on *Eciton* in Brazil (type) and Ecuador; *Laboulbenia formicarum* Thaxt. on *Formica*, *Lasius*, *Myrmecocystus*, *Prenolepis*, and *Polyergus* in Canada, the USA (type), France, Portugal, and Spain; the recently described *Rickia lenoirii* Santam. on *Messor* in France and Greece (type); and *Rickia wasmannii* Cavara on *Myrmica* in Austria, Bulgaria, Czech Republic, France, Germany, Hungary, Italy, Luxembourg, the Netherlands, Poland, Rumania, Slovakia, Slovenia, Spain, Switzerland, and the United Kingdom (Tavares, 1985; Rossi, 1991; Espadaler & Santamaría, 2012; Haelewaters, 2012; Báthori et al., 2014; Santamaría & Espadaler, 2014; Witek et al., 2014). See Haelewaters et al. (2015) for an overview of all published records of *R. wasmannii*, including all *Myrmica* host species.

In a previous account (Haelewaters et al., 2015) we presented a mass infection of *Rickia wasmannii* Cavara on *Myrmica sabuleti* Meinert, 1861, with 38% out of 3876 collected workers being infected. After comparing our data to different studies from Hungary, Romania, and Slovakia, where *M. scabrinodis* Nylander, 1846 often was the only or main host (Tartally et al., 2007; Bezděčka & Bezděčková, 2011; Csata et al., 2013), we suggested that infection with *R. wasmannii* shows geographical variation; the dominant host species varies across regions. In this paper we provide details about the distribution of *R. wasmannii* on host species in our study site and variation of infection over habitats and over time.

Material and methods

Three series of pitfall traps were placed in a study area that spans somewhat less than 1 km², situated east of Maastricht (Limburg, the Netherlands) near the border with Belgium. The pitfall traps of the first two series were filled with a formol so-

lution (3%), while those of the third series were filled with a saturated salt solution. A lid was placed above each trap to prevent rain from falling in. For an overview of the study area, see fig. 1.

The first series of 9×5 pitfall traps was placed at the verge of a railroad. Here, a mosaic of rough and grassy vegetation to open calcareous grassland was present. These traps were emptied monthly year-round, starting February 4 to December 10 2013.

The second series, 11×5 traps, was placed in an adjacent grassland dominated by *Arrhenatherum elatius*, *Bromus erecta*, *Origanum vulgare*, *Plantago lanceolata*, *Poa trivialis*, and *Rhinanthus alectorolophus*. This grassland was an agricultural field until 1986; from that year onwards nature restoration was performed. The only management has been haymaking once a year. Throughout the years, a thin and species-rich vegetation has developed on this field. A small patch with more rough vegetation was in this series as well. These traps were sampled from May 11 to July 7 2013.

Students at the University of Amsterdam (UvA) sampled a third series of 9×36 pitfalls, which were placed in diverse habitats, 36 pitfall traps per habitat: forest on hill plateau, forest on hill's slope, forest edge, moist forest, felling area, calcareous grassland, agricultural field edge, hollow road, and verge of the above-mentioned railway track. This set of traps was operational in the period June 17–21 2013.

Ants were identified to species level using Boer (2010). Screening for Laboulbeniales thalli was done at $45\times$ magnification with a Euromex Z-1740H stereomicroscope (Arnhem, the Netherlands). For microscopy techniques dealing with Laboulbeniales mountings, see Haelewaters et al. (2015).

Statistical analysis

To find seasonal patterns in the first (year-round) series of pitfall traps, we analysed the fractions of infected workers of *M. sabuleti* and of *M. scrabinodis* separately with Generalized Linear Mixed Models (GLMM, Littell et al., 2006), using procedure GLIMMIX of the SAS[®] software system, version 9.3. In the GLMM a quasi-binomial distribution for the number of infected ants was assumed, using a logit link function and allowing overdispersion compared to the binomial distribution. We assumed random effects for pitfalls (allowing infected fractions to vary from pitfall to pitfall) and fixed effects for time (allowing infected fraction to change over the season). To model the time effect we defined a time factor with three levels: period 1 (Feb–May, hereafter referred to as *spring*), period 2 (June–Aug, *summer*), period 3 (Sep–Oct, *autumn*).

Due to limited numbers of pitfalls, observations, and infected individuals for pitfall trap series 2 and 3, we limited statistical analysis in these cases to descriptive statistics only.

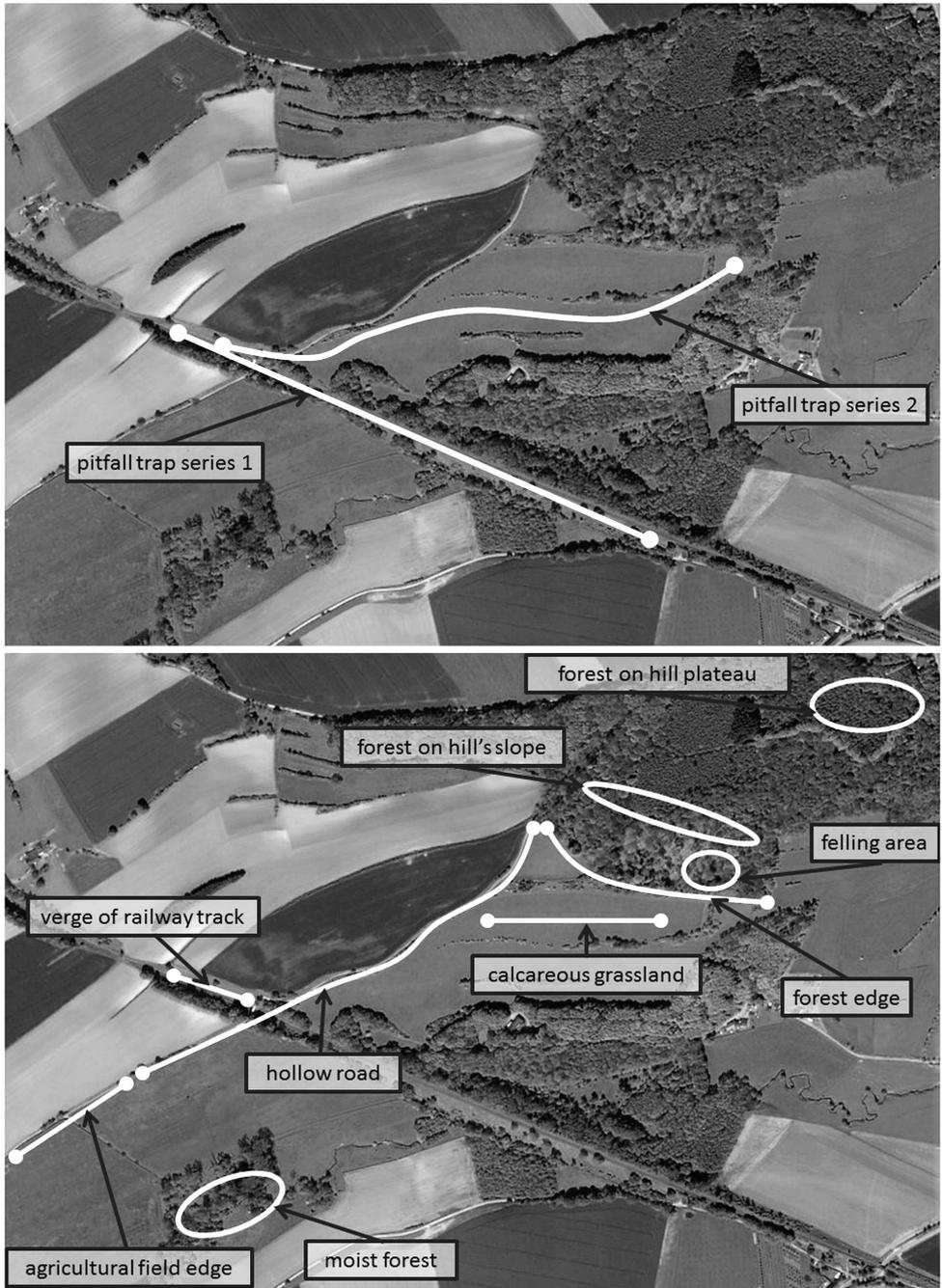


Figure 1. The study site with all the sampling localities indicated. Trajectories (lines) and areas (ovals) were sampled with pitfall traps during three inventories. The separate inventories mentioned in the text are shown. Above, pitfall trap series 1 and 2. Below, pitfall trap series 3.

Results

Pitfall traps series 1

Table 1A shows the numbers of workers (total and infected) for both *M. sabuleti* and *M. scabrinodis*, sampled year-round from the first series of pitfalls. 2093 workers of *M. sabuleti* were collected, 1326 of which were infected with *R. wasmannii* (63.4% of the sampled population). Each time the traps were emptied, high parasite prevalence was found ($\geq 49\%$).

Myrmica scabrinodis was found in the traps less frequently ($n = 226$); the average parasite prevalence was 34.1% ($n = 77$). The sample from April 10 to May 10 had the highest prevalence (89%, $n = 38$).

For *M. sabuleti*, we found significant differences between periods ($F_2 = 4.18$, $P = 0.023$; table 1B). The estimated mean infection rate was declining over the seasons: 67% during springtime, 46% during the summer and 40% in autumn (table 1B). We found extremely large variation in infection rate between pitfall trap series, which is most likely due to the presence of nests with high or low infection rate near a series. Furthermore, infection rates varied more than could be accounted for by the binomial distribution. The same declining trend over the seasons was observed for *M. scabrinodis*, but the differences were not significant now ($F_2 = 2.38$, $P = 0.1265$). Again, a very large variation in infection rates between pitfall trap series was found.

Pitfall traps series 2

Only nine of 657 *M. sabuleti* workers (1.4%) bore thalli of *R. wasmannii* (table 1C), although the pitfall traps in this second series were located near the verge of the railroad where a parasite prevalence of 63% was found (pitfall traps series 3, UvA3). Six of the nine infected *M. sabuleti* were collected from trap M9, in which most of *M. scabrinodis* workers – all uninfected – were found ($n = 100$).

Pitfall traps series 3

The third series of pitfall traps provided more specific information about the distribution of both the ant species and their infection with *R. wasmannii* over different habitats (table 1D).

Myrmica sabuleti was found in all habitats except one (felling area). In five locations nine or more workers were found, including infected ones in each occasion. The highest number of *M. sabuleti* specimens ($n = 903$) was recorded at a hollow road, of which 22 were infected with *R. wasmannii* (2.4%). The highest number of infected individuals was found at the verge of a railroad: 91% ($n = 144$), a location close to the pitfall traps of series 1. The infection rate in the same period here was 77%.

In these traps, few specimens of *M. scabrinodis* were found (total $n = 100$). With three locations yielding no specimens at all (forest on hill plateau, felling

Table 1.

Data from the three series of pitfall traps showing total and infected number of collected workers, and infection prevalence, for *M. sabuleti* and *M. scabrinodis*. (A, B) Data from the first series of 54 pitfall traps: A, parasite prevalence per sample; B, observed counts and predicted fractions with n = total number of workers, k = observed number of infected workers, p = predicted fraction of infected workers (\pm approximated standard error) based on GLMM. (C, D) Total and infected number of *Myrmica sabuleti* and *M. scabrinodis* ants, collected in the second, respectively third series of pitfall traps.

A	<i>Myrmica sabuleti</i>			<i>Myrmica scabrinodis</i>		
	Total #	Infected #	Infection %	Total #	Infected #	Infection %
Series 1: samples						
February 4-April 10	50	40	80	9	0	0
April 10-May 10	318	224	70	38	34	89
May 10-June 12	401	234	58	43	7	16
June 12-July 10	283	219	77	35	23	66
July 10-August 14	289	185	64	44	9	20
August 14-September 11	274	135	49	43	2	5
September 11-October 16	251	175	70	10	2	20
October 16-December 10	227	114	50	13	0	0
TOTAL	2093	1326	63.4	226	77	34.1
B	<i>Myrmica sabuleti</i>			<i>Myrmica scabrinodis</i>		
	n	k	p (\pm SE)	n	k	p (\pm SE)
Spring	368	255	0.67 (\pm 0.14)	38	34	0.32 (\pm 0.28)
Summer	973	534	0.46 (\pm 0.14)	121	31	0.12 (\pm 0.11)
Autumn	525	308	0.40 (\pm 0.14)	63	4	0.05 (\pm 0.06)

Table 1.
(Continued.)

C	<i>Myrmica sabuleti</i>			<i>Myrmica scabrinodis</i>		
	Total #	Infected #	Infection %	Total #	Infected #	Infection %
Series 2: 11 × 5 traps each						
M1	4	0	0	14	0	0
M2	7	0	0	43	0	0
M3	87	0	0	27	0	0
M4	7	1	14	27	0	0
M5	197	0	0	23	0	0
M6	67	1	1.5	45	0	0
M7	90	0	0	6	0	0
M8	68	1	1.5	62	0	0
M9	24	6	25	100	0	0
EN	21	0	0	10	0	0
ES	85	0	0	5	0	0
TOTAL	657	9	1.4	362	0	0
D	<i>Myrmica sabuleti</i>			<i>Myrmica scabrinodis</i>		
	Total #	Infected #	Infection %	Total #	Infected #	Infection %
Series 3: 9 × 36 traps each						
UvA1	1	0	0	0	0	0
UvA2	9	7	78	7	0	0
UvA3	27	17	63	2	0	0
UvA4	1	0	0	1	0	0
UvA5	0	0	0	0	0	0
UvA6	78	1	1.3	74	1	1.4
UvA7	1	0	0	6	0	0
UvA8	903	22	2.4	10	1	10
UvA9	144	131	91	0	0	0
TOTAL	1164	178	15.3	100	2	2

area, verge of railway track), only in one trap were more than ten specimens of this species collected (calcareous grassland; $n = 74$). Infection with *R. wasmannii* was found only twice, one in the calcareous grassland, the other at a hollow road.

Discussion

Infection and age of host

During our study, infections with *R. wasmannii* were only found in workers, but gynes were always uninfected. Members of the Laboulbeniales occur almost exclusively on adult hosts; infections of pre-imaginal stages are extremely rare and have only been observed on certain hosts (cockroaches, termites and ants). Baumgartner (1934) mentions infected larvae and nymphs in an artificial ant nest. Integuments with thalli are lost when nymphs and adults emerge (observed by Baumgartner, 1934). One study mentions infected dealate (old) gynes (Tartally et al., 2007).

Alate (young) gynes are never infected although they frequently encounter intraspecific workers, who regularly “lick” and feed the gynes. Potential contributing factors are the workers’ cleaning licking behavior and the surface ultrastructure of the young developing integument, which might keep the ascospores from successful attachment.

Freshly hatched workers, which can be recognized by their pale yellowish color, were found uninfected by Csata et al. (2014); in our study site in the Netherlands they were seldom infected. We hypothesize that infection with *R. wasmannii* starts at the moment the cuticle has hardened. This is consistent with observations of *Harmonia axyridis* (Pallas, 1773) (Coleoptera, Coccinellidae) on a lighted white sheet at night in De Kaaistoep (Tilburg, the Netherlands, van Wielink & Spijkers, 2013). Most of the *H. axyridis* collected at night with soft elytra were not infected with *Hesperomyces virescens* Thaxt. (Laboulbeniales) (P. van Wielink, personal communication). Winter aggregations had on average higher infection rates than specimens collected at night (Raak-van den Berg et al., 2014). Again, also in the case of *H. virescens* we assume that infection only starts on individuals with a hardened cuticle.

Infection and time of the year

Taken over three periods throughout the year (spring, summer, autumn; table 1B), parasite prevalence significantly declined in *M. sabuleti*. While the same declining trend was present for *M. scabrinodis*, the differences observed were not significant. This is probably due to the smaller sample sizes of *M. scabrinodis* workers. Below we offer possible explanations for the observed differences in parasite prevalence between the different samples in the first series of pitfall traps.

For both *M. sabuleti* and *M. scabrinodis*, the highest parasite prevalence was observed after the winter. Infection with Laboulbeniales is often seen as a sexually transmitted disease (e.g. Welch et al., 2001), although there are examples where

the occurrence of thalli on the host's body is not (only) explained by mating contact. *Harmonia axyridis*, a common host to *Hesperomyces virescens*, hibernates in dense aggregations. It is shown that this direct contact between hosts is an important mechanism of transmission of the fungus (Riddick & Schaefer, 2005; Riddick, 2006; Nalepa & Weir, 2007). Cockroaches live together in large numbers (Tavares, 1985). Individuals continuously groom and make contact with each other providing excellent conditions for transmission of spores, exemplified by (extremely) high parasite prevalences for *Herpomyses stylopygae* Speg. (Richards & Smith, 1955) and *H. chaetophilus* Thaxt. (Wang et al., in press) on their respective hosts. Of course, worker ants do not mate and thus get infected by other contact. Supposedly, *Rickia* is transmitted while uninfected workers lick infected workers. This is supported by observational data from our study: all infected workers bore thalli on their head, while less infected workers had thalli mostly on the frontal side of the head (fig. 2; data not shown, manuscript in preparation).

In ants, during wintertime, the aboveground activity decreases and individuals aggregate in dense clusters in their nests. Ants make many contacts with each other, mainly in the context of allogrooming. Increased allogrooming, which is important for cleaning areas that cannot be accessed during autogrooming (Csata et al., 2014), may be an important reason for the high parasite prevalence observed after winter. Additionally, in this time of the year dead infected workers are not deposited outside



Figure 2. The head of a *Myrmica sabuleti* worker, heavily infected with *Rickia wasmannii*. Photograph: Theodoor Heijerman.

the nest (which is done in spring and summer, e.g. Heinze & Walter, 2010), and this might also contribute to the high infection rate at the end of the winter.

In addition, we suggest that autogrooming contributes to the spread of ascospores of *R. wasmannii* over the ant worker body, and thus to higher thallus densities over the course of winter. In *H. axyridis* ladybirds, autogrooming results in higher thallus densities of *H. virescens* on older hosts (Raak-van den Berg et al., 2014). Csata et al. (2014) found the frequency of both auto- and allogrooming to be higher in infected nests compared to uninfected nests (artificial nests under laboratory conditions). This behavioral change, whether or not parasite-induced, increases both parasite prevalence and thallus densities, two parameters used to measure population dynamics in Laboulbeniales (e.g. De Kesel, 1995, 2011; Riddick & Schaefer, 2005; Raak-van den Berg et al., 2014).

The decrease in parasite prevalence in the sample from May 10 to June 12 followed the emergence of a new uninfected cohort of workers, originated from overwintering larvae (Kipyatkov, 1993). In June/July infection rate rose, possibly because the new workers were infected by allogrooming.

After the sample from June 12 to July 10, parasite prevalence decreased again, which can be explained by the emergence of yet another new cohort of workers, from eggs that were laid in spring (Kipyatkov, 1993), in combination with some effect of infection on the lifespan of ants. Csata et al. (2014) showed that in laboratory conditions on average 15% of workers died in uninfected nests, while 41% of workers died in infected nests. Moreover, infected workers lived significantly less long than uninfected workers in the laboratory experiment. Additionally, it was shown that infected *Myrmica* workers need more water, which could explain that heavily infected hosts die in drier periods (data on *M. scabrinodis* workers, Báthori et al., 2015). This might contribute to the decreasing prevalence we observed in summer.

It can be concluded that variations in parasite prevalence are the result of 1) how much time the workers spend in the nest, mainly depending on the season; 2) mortality, including that induced by *Rickia*; and 3) the emergence of cohorts of uninfected, freshly hatched workers. The effect of three phenomena remains unclear: necrophoresis, social withdrawal, and nest population relocation. Necrophoresis refers to the removal of dead individuals from the ant nest. Dead ant bodies are generally left behind just outside the exit of the nest (Hölldobler & Wilson, 1990). This prevents accumulation of infected material within the nest but at the same time increases the chance of infection outside of the nest. Social withdrawing of dying workers, to die in seclusion, might be an effective way of minimizing the risk of infecting others, in case of infection (Heinze & Walter, 2010). The effect is similar to that of necrophoresis. One question remains: How many withdrawn infected workers did we collect during our study? Most species of *Myrmica* relocate their nest once to several times a year (Radchenko & Elmes, 2010). It is unclear whether or not this is beneficial in reducing infection with *R. wasmannii*, although we have observed highest parasite prevalence in sites where *M. sabuleti* was most densely populated. In non-overpopulated sites nest relocations will happen less likely to

sites with previously infected nests, where potentially dead infected ant bodies may have been left behind.

Distribution over habitats

The third series of pitfall traps indicates that *R. wasmannii* can occur across several habitats, from moist, cool forests to dry, warm calcareous grasslands. *Rickia wasmannii* occurs on ant species that prefer moist habitats as well as ones that prefer dry habitats (Elmes et al., 1998), with respectively *M. gallienii* and *M. specioides* as the extremes. *Myrmica gallienii*, *M. specioides*, *M. spinosior*, and *M. vandeli* have been found infected with *R. wasmannii* in only one or two countries (Tartally et al., 2007; Espadaler & Santamaría, 2012; Csata et al., 2013; reviewed in Haelewaters et al., 2015). This may have to do with the restricted and/or scattered distribution of these ant species (Seifert, 2007; Radchenko & Elmes, 2010). *Myrmica ruginodis* most often occurs in shady habitats (Boer, 2010), as was the case in our study. Here, other *Myrmica* ants are absent or rare, so contact with infected *M. sabuleti* and *M. scabrinodis* nests is lacking. In our study only one specimen of *M. ruginodis* was infected, and this specimen was caught in a series of traps where also 67 specimens of *M. sabuleti*, of which 16 infected with *R. wasmannii*, were caught. For Romania, the only other country where infected workers of *M. ruginodis* have been found, no parasite prevalence information is available (Csata et al., 2013).

The remarkably lower parasite prevalences of *Rickia wasmannii* on *M. sabuleti* (9%) and *M. scabrinodis* (0%) in the second series of pitfalls suggest that relatively young habitats (this site was converted from an agricultural field to natural grassland in 1986) have lower rates of infection compared to older sites. There probably is a build-up of infection by *Rickia* over time through worker contact.

Conclusions

In our study site in the Netherlands, *Rickia wasmannii* proved to be a common ectoparasite. Infections are always found on workers, while gynes are uninfected. Young, freshly hatched workers are seldom infected. This is in agreement with observations of *H. virescens* on *H. axyridis* and led us to hypothesize that infection with Laboulbeniales only starts at the moment the cuticle has hardened. We found that parasite prevalence varies for different ant host species (Haelewaters et al., 2015), and for colonies of ants of different ages, resulting in changes in infection rate in the course of the year. Occurrence of *R. wasmannii* does not differ across different habitat types, although ants in the sampled young habitat are less infected. Main hosts and temporal and spatial patterns of the ectoparasite might vary across regions (e.g., Haelewaters et al., 2015), making it interesting to conduct more, collaborative studies at different locations within the range of *R. wasmannii*.

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