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RESEARCH ARTICLE

Multiple fungal hosts in an ant microcosm: *Rickia wasmannii* infecting larvae, winged sexuals, and arthropod associates of *Myrmica* ants

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Abstract

Parasite infections beyond the traditionally reported host range may point to previously unrecognized host–parasite dynamics. In this study, we investigated the host range of the ectoparasitic fungus *Rickia wasmannii* (Ascomycota, Laboulbeniales), which has to date been documented mainly on the cuticle of *Myrmica* ant workers. We first assessed the prevalence of infections in workers and then screened for infections in wingless and winged sexuals, larvae, and arthropod associates from 41 nests across four *Myrmica* species. Seven colonies were infected, with all workers carrying thalli. Wingless (reproductive) queens, winged (virgin) queens, and males were detected in three of these colonies and were all infected (5, 40, and 55 individuals, respectively), representing the first records of *R. wasmannii* on winged sexuals. A small proportion of larvae (18 out of 340 specimens) and mites (24 out of 1951 individuals) in the infected colonies carried thalli, as well as one individual of the specialized myrmecophilous beetle *Lomechusa emarginata* (Staphylinidae). We observed a single mature *R. wasmannii* thallus on a larva, while all thalli on arthropod associates were immature. Overall, our findings indicate that *R. wasmannii* can infect multiple *Myrmica* castes within the shared nest microhabitat. Infections on larvae and arthropod associates also occurred occasionally, but were characterized by low thallus densities and/or the absence of mature thalli.

Keywords Acari · Arthropod–fungal associations · Formicidae · Myrmecophile · Parasite · Staphylinidae

Communicated by Enikő Csata

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Introduction

Ant colonies host a remarkable diversity of associated organisms in their nests, ranging from microorganisms such as bacteria, fungi, and protists to arthropod associates and even some vertebrates (Hölldobler and Kwapich 2022; Parmentier et al. 2020). The favorable conditions of ant nests help explain this diversity and specialization. Nests are nutrient-rich, buffered from external abiotic fluctuations, and generally have lower pathogen loads compared with surrounding soil (Schär et al. 2015; Véle and Holuša 2017). Colonies also effectively deter larger predators. Together, these factors create stable, resource-rich microenvironments that attract and sustain a wide range of specialized symbionts. Within this diverse community, host specialization varies widely; some associates are restricted to a single ant species or genus, while others occur across multiple subfamilies and some symbionts are even found with both ants and non-ant taxa (Molero-Baltanás et al. 2025; Parmentier et al. 2020). Host specialization plays a pivotal role in the ecology and evolution of ant-associated organisms, often aligning with their degree of trait specialization and with profound implications for their population and colonization dynamics (von Beeren et al. 2018). The effects of these organisms on their hosts also span a broad spectrum, from neutral to mutualistic or parasitic. For example, fungi and bacteria associated with leafcutter ants contribute to food digestion and defense against pathogens (Hölldobler and Wilson 2011), whereas parasites and pathogens can reduce host fitness by extracting resources or even killing the host (Hölldobler and Kwapich 2022). Despite this apparent richness, for many ant-associated organisms both host specificity and the nature of their interactions remain poorly understood or are inferred from limited evidence.

The inhabitants of ant nests form highly integrated natural communities, characterized by a variety of intraspecific and interspecific interactions (Parmentier et al. 2016). The most prominent interactions occur within the ant colony, where workers, males, winged (virgin) queens, wingless (reproductive) queens, and larvae engage in a tightly connected behavioral network, including food exchange, transport, and grooming (Hölldobler and Wilson 1990). Associated arthropods, commonly known as myrmecophiles, interact directly and indirectly with colony members, each other, and the shared nest material (Parmentier et al. 2018). Very close heterospecific interactions occur between ants and some myrmecophiles, such as *Maculinea* caterpillars and rove beetles (*Lomechusa*, *Claviger*), which are treated as colony members through grooming, transport, and feeding (Hölldobler and Kwapich 2022). Parasites, including myrmecophilous mites and certain fungi, also closely interact with both the host colony and the nest environment. These intimate interactions facilitate the transmission of pathogens and viruses across castes and potentially between the host and associated arthropods. Although such heterospecific spillovers are poorly understood in ant nests, research in other social insects, particularly honeybees, shows that viruses, bacteria, and fungi can be transmitted to associated arthropods, including the small hive beetle and *Varroa* mites (Cilia et al. 2018; Eyer et al. 2009; Nanetti et al. 2021).

Rickia wasmannii (Laboulbeniales) is considered as a fungus strictly associated with *Myrmica* ants. As an ectoparasite, it completes its life cycle on the cuticle of *Myrmica* ants, with 10 different species recorded as hosts thus far (Haelewaters et al. 2015a; Báthori et al. 2017a). In contrast to those Laboulbeniales that form a haustorium, i.e., a root-like structure that penetrates the host cuticle and accesses the haemocoel, *R. wasmannii* lacks such a structure and attaches superficially but firmly to its host by means of a foot cell (Tragust et al. 2016). Nevertheless, *R. wasmannii* significantly affect its ant hosts, leading to increased water uptake (Báthori et al. 2015), reduced boldness and aggression (Báthori et al. 2017b), and greater acceptance of outsider ants and other arthropods (Csata et al. 2017). Infected ants also exhibit thinner cuticles, reduced body sizes, and increased allogrooming (Csata et al. 2014, 2018; Csősz et al. 2021). It is hypothesized that the absence of a haustorium may allow for a broader host range, as the fungus does not need to develop specific adaptations to bypass the host's defense system specificity and is not dependent on certain nutrients from the host body (Haelewaters et al. 2019, 2022). Direct body contact is likely required for heterospecific transmission, as the ascospores are sticky, not airborne, and short-lived (Huldén 1983; De Kesel 1995; Kesel 1996). The idea of "ecological specificity" (Scheloske 1969) suggests that fungi may shift between unrelated hosts occupying the same habitat (Benjamin 1971; De Kesel and Haelewaters 2014). Ascospores of *R. wasmannii* could then develop on non-*Myrmica* arthropods if they co-occur within the nest micro-environment of a *Myrmica* host. Supporting this idea, Pfliegler et al. (2016) demonstrated that *R. wasmannii* can infect myrmecophilous mites and a fly larva (*Microdon*) in a *Myrmica* nest. Given the limited data available, these patterns remain speculative, and further experimental work is needed to clarify the host range of *Rickia* and the factors influencing it.

Rickia wasmannii infections have so far been reported mainly on *Myrmica* workers (Bezděčka and Bezděčková 2011; Haelewaters et al. 2015b), whereas recorded infections of wingless queens are rare (Tartally et al. 2007) and those of winged queens are non-existent. Males within nests have likewise not been recorded as carriers, and larvae are known to be carriers from only a single anecdotal report (Baumgartner 1934).

This study aims to assess whether *R. wasmannii* is strictly specific to *Myrmica* workers, or if it can also infect and develop to maturity on other castes and non-*Myrmica* arthropod hosts within the *Myrmica* nest microhabitat. Therefore, we screened 41 colonies from four *Myrmica* species together with their arthropod associates. We examined the presence of the thalli on ant workers, wingless and winged queens, males, and larvae, as well as the arthropod associates.

Materials and methods

Site description

Myrmica ant nests were collected over 9 days from May to October 2022 at a 32-ha site in Gulpen-Wittem (50°49'38.30"'N 5°54'35.37"'E), within the Geuldal valley in the southern province of Limburg, The Netherlands (Table 1). The site was chosen because high densities of *Rickia* infected *Myrmica* ants had been observed previously (De Kesel et al. 2016; Haelewaters et al. 2015a, b). A total of 41 *Myrmica* nests were collected from different habitats in this site: 17 in calcareous grasslands, 15 in thermophilic pioneer vegetation on calcareous bedrock, 5 in a forest patch, and 4 in a hydrophilous tall herb fringe. Nests were excavated thoroughly using a trowel and rake. In many cases, only portions of nests could be collected due to the type of terrain (stony soil) or inaccessible nest parts. The openings of all sampled nests were spaced at least 1.5 m apart to ensure different nests were sampled. All nests were stored in stable room temperature environments with little to no light before sorting in the lab. For each nest, we screened all individuals and associated organisms from the collected portion. We note that winged sexuals are present in *Myrmica* nests only for a short period in summer, and that many of our sampled nests were collected before or after this period.

Sorting and screening of colonies

The day after collection, the nest material, along with the *Myrmica* colony and associates, was spread in a large tray. To improve efficiency, moist gypsum slabs were added to the tray, under which ants and associates gathered. Ants were collected using tweezers and a mouth-operated aspirator, then stored in separate vials with 70% ethanol according to their caste: males, wingless (reproductive) queens, winged (virgin) queens workers, and larvae. Larger associates were sorted into 70%-ethanol vials based on their broad taxonomic group. The nest material was then placed in Berlese funnels connected to ethanol vials for two days to sample smaller associated arthropods, such as springtails and mites. Obligate myrmecophilous isopods, springtails and beetles were identified to species level, mites were identified to order level (Parasitiformes, Acariformes), or, if possible, to suborder (Mesostigmata, Oribatida, Astigmata) or Cohort level (Uropodina) (André and N'Dri 2013). Mites contain obligate and facultative associates, but identification at the species level is required to infer their degree of association.

Screening for the presence of *R. wasmannii* was performed under a Leica MZ125 microscope at 10–160x magnification, with the aid of an Euromex Fiber Optic Light Source EK-1. Arthropod associates and larvae were screened only for those nests with infected *Myrmica* workers. During the screening, sorting was conducted based on whether specimens were infected or uninfected. Arthropod associates and larvae were further divided based on whether fungal thalli were mature. At this stage, the sexual ants were formally classified as: males, winged (virgin) queens, and wingless (reproductive) queens. After the screening the identification of ants was carried out at the species level based on Boer (2015).

Microscope slide preparation

Microscope slides were prepared of thalli from *R. wasmannii* present on infected workers and larvae to assess maturity and for long-term storage. A double coverslip technique was used to mount thalli on permanent slides (Liu et al. 2020). First a droplet of Hoyer's medium was placed on a 22 × 22 mm coverslip. Thalli were taken at the point of attachment with a needle that had been dipped in the Hoyer's medium. Once detached from the host, thalli were transferred into the droplet on the coverslip and rearranged in one or multiple rows. Next, a drop of Amann's medium was placed on the 18 × 18 mm coverslip, which was then turned upside-down and carefully placed sideways onto the 22 × 22 mm coverslip. The corners of the small coverslip were sealed to the underlaying larger coverslip with nail polish. Finally, Solakryl BMX medium (Ento Sphinx s.r.o.) was placed onto the microscope slide. The coverslips were flipped, i.e., the small coverslip facing down, and placed sideways in the Solakryl BMX medium. The slides dried overnight under a fume hood. These slides were then photographed using a Nikon DS-Fi3 microscope mounted on a Nikon Eclipse Ni-U microscope. Slides are permanently housed in the Herbarium Universitatis Gandavensis (GENT), Ghent, Belgium. Maturity of thalli was determined based on characteristics of the perithecium. Only thalli with a fully grown perithecium measuring 48–55 µm in length (De Kesel et al. 2016) and with visible two-celled ascospores inside were considered mature.

Table 1 Infection prevalence of *Rickia wasmannii* in different ant castes

colony	sampling date	workers	wingless queens (reproductive)	winged queens (virgin)	males	larvae
Myrmica sabuleti						
Myr_WE_008	03/06/2022	**94** (94)	-	-	-	-
Myr_WE_021	21/07/2022	**133** (133)	-	**40** (40)	**42** (42)	0 (95)
Myr_WE_040	06/10/2022	**233** (233)		-	-	**11** (176)
Myr_WE_041	06/10/2022	**441** (441)		-	--	-
Myr_WE_007	03/06/2022	0 (38)	-	-	-	-
Myr_WE_018	21/07/2022	0 (48)	-	-	-	-
Myr_WE_019	21/07/2022	0 (137)	-	-	0 (2)	-
Myr_WE_020	21/07/2022	0 (7)	-	-	-	-
Myr_WE_022	21/07/2022	0 (31)	-	-	0 (1)	
Myr_WE_024	28/07/2022	0 (495)	-	0 (1)	-	0 (479)
Myr_WE_032	11/08/2022	0 (147)	-	-	-	0 (36)
Myr_WE_034	19/08/2022	0 (569)	-	-	0 (6)	0 (151)
Myr_WE_036	19/08/2022	0 (129)	-	-	-	0 (49)
Myr_WE_037	19/08/2022	0 (305)	-	-	-	0 (75)
Myrmica scabrinodis						
Myr_WE_003	03/06/2022	**207** (207)	-	-	-	0 (14)
Myr_WE_038	25/08/2022	**85** (85)	**3** (3)	-	**13** (13)	
Myr_WE_039	29/09/2022	**102** (102)	**2** (2)	-	-	**7** (55)
Myr_WE_035	19/08/2022	0 (305)	-	0 (15)	-	0 (23)
Myrmica ruginodis						
Myr_WE_006	03/06/2022	0 (21)	-	-	-	-
Myr_WE_011	03/06/2022	0 (443)	0 (3)	-	-	-
Myr_WE_023	28/07/2022	0 (89)	-	-	-	0 (8)
Myrmica rubra						
Myr_WE_001	03/06/2022	0 (168)	0 (1)	0 (1)	-	-
Myr_WE_002	03/06/2022	0 (126)	-	-	-	-
Myr_WE_004	03/06/2022	0 (118)	-	-	-	-
Myr_WE_005	03/06/2022	0 (184)	-	-	-	-
Myr_WE_009	03/06/2022	0 (156)	-	0 (1)	-	-
Myr_WE_010	03/06/2022	0 (448)	0 (4)	-	-	-
Myr_WE_012.1	03/06/2022	0 (145)	-	-	-	-
Myr_WE_012	14/07/2022	0 (170)	-	-	-	-
Myr_WE_013	14/07/2022	0 (523)	-	-	0 (17)	-
Myr_WE_014	14/07/2022	0 (202)	-	-	-	-
Myr_WE_015	14/07/2022	0 (232)	-	0 (1)	0 (2)	-
Myr_WE_016	14/07/2022	0 (157)	-	-	-	-
Myr_WE_017	14/07/2022	0 (80)	-	-	0 (2)	-
Myr_WE_025	28/07/2022	0 (902)	0 (1)	-	-	-
Myr_WE_026	28/07/2022	0 (359)	0 (1)	-	-	0 (319)
Myr_WE_028	11/08/2022	0 (448)	-	-	-	0 (297)
Myr_WE_029	11/08/2022	0 (110)	-	-	-	0 (201)
Myr_WE_030	11/08/2022	0 (304)	-	-	-	0 (47)
Myr_WE_031	11/08/2022	0 (20)	-	-	-	0 (7)
Myr_WE_033	19/08/2022	0 (153)	-	-	-	0 (153)

Numbers in parentheses indicate the total number of individuals examined, and the number before the parentheses shows how many were infected

Results

We observed thalli on workers, winged and wingless queens, larvae, and arthropod associates (myrmecophiles). The thalli observed in all these hosts were consistent with those of *Rickia wasmannii*, matching descriptions from previous studies, except for the absence of perithecia in most of the thalli (Cavara 1899; De Kesel et al. 2016).

Ants

We sampled colonies of four species of *Myrmica* ants: 20 colonies of *M. rubra*, 14 colonies *of M. sabuleti*, 4 colonies *M. scabrinodis*, and 3 colonies of *M. ruginodis* (Table 1). Worker infections were found in 7 of the 41 colonies belonging to two *Myrmica* species: 4 of 14 colonies of *M. sabuleti* and 3 of 4 colonies of *M. scabrinodis* were infected (Table 1). Infections were spatially spread across the study site, but with a notable clustering of four infected colonies in the center of our study site (Fig. 1). All workers consistently carried *R. wasmannii* thalli in the seven infected colonies (Table 1). Five wingless queens were found in two of these colonies and they were all infected. Males ($N=55$) were found in two colonies with infected workers and winged queens ($N=40$) were found in one colony with infected workers. These winged sexuals also reached 100% infection (Table 1). Infected larvae were found in two colonies with infected workers: 11 out of 176 larvae carried thalli in a *Myrmica sabuleti* colony (Myr_WE_040), and 7 out of 55 larvae in the *M. scabrinodis* colony (Myr_WE_039) were infected (Table 1). Most infected larvae supported a single thallus. Two larvae supported two thalli, and in one case, one of the thalli was mature (Fig. 2).

Associates

A total of 2250 invertebrates associated with the 7 infected *Myrmica* colonies (Table 1) were screened for the presence of *R. wasmannii* (Table 2). The most abundant group were mites (1951 specimen), which we categorized in higher-level taxa. A total of 1744 of the screened invertebrates belonged to the mite suborder Acariformes, including oribatid mites (suborder Oribatida), dust mites and phoretic deuteronymphs (suborder Astigmata), and other acariform mites of which the suborder could not be determined. Five individuals of acariform mites (distributed across two *M. sabuleti* nests and one *M. scabrinodis* nest, see Table 2) showed clear signs of infection. All of these were phoretic deutonymphs of an as-yet unidentified astigmatid mite. The second-most common group of associates was the mite suborder Mesostigmata (207 specimens). Within this group, members of the Uropodina cohort were frequently observed. They were typically found attached to ant workers. Among these mites, 17 (16 in nest Myr_WE_021 and 1 in nest Myr_WE_039) were infected with *R. wasmannii*. One of these uropodid infected mites was identified to species level as *Trichocylliba comata* (based on Mašán 1999). In addition, two individuals from the predatory mesostigmatid family Laelapidae (cohort Gamasina) were found to be infected (nest Myr_WE_021). No more than two thalli were detected on any single mite. Thalli on mites were not mature. Most of the other collected arthropods were facultative associates, such as millipedes, centipedes, spiders, and springtails. None of these were infected. Obligate non-mite myrmecophiles were the ant springtail *Cyphoderus albinus*, the ant isopod *Platyarthrus hoffmannseggii* and the rove beetle *Lomechusa emarginata*. Individuals of the isopod

Fig. 1 Locations of sampled areas in the Geuldal Valley in the Gulpen-Wittem municipality. Each color represents a different *Myrmica* species and filled triangles represent colonies infected with *Rickia wasmannii*

Fig. 2 **A** *Lomechusa emarginata* (D. Haelew. 4799) with thalli of *Rickia wasmannii* visible on its antennae, pronotum, elytra, and metasternum (a single thallus). **B** An infected mite. **C** A developing thallus of *R. wasmannii* from a *Myrmica scabrinodis* larva (slide GENT: GENTFL01266, nest Myr_WE_039). **D** A mature thallus of *R. wasmannii*, with fully developed perithecium that contains two-celled ascospores, from a M. *scabrinodis* larva (slide GENT: GENTFL01268, nest Myr_WE_039)

P. hoffmannseggii (41 screened individuals) and springtail *C. albinus* (33 screened individuals) did not carry thalli in infected *Myrmica* nests (Table 2). The myrmecophilous beetle *Lomechusa emarginata*, which was only sampled once, was found to be heavily infected with thalli on different body parts (antennae, thorax, head, elytra, and anterior legs) in a *M. scabrinodis* nest (Fig. 3), but no mature thalli could be detected.

The number and extent of infections among associate species may be underestimated. Some individuals lacked visible thalli but exhibited small black dots, which likely represent the foot cells of developing *Rickia* thalli. For example, In the nest of colony Myr_WE_003, three mesostigmatid predatory mites displayed black dots, though the origin of these markings remains uncertain.

Discussion

Our results indicate that *R. wasmannii* infection in *Myrmica* nests is more complex than previously assumed, involving not only workers but also sexuals, larvae, and nest-associated arthropods. Within *Myrmica* colonies, *R. wasmannii* thalli have traditionally been observed primarily on the worker caste, with other screened castes reported as uninfected (Haelewaters et al. 2015b). However, Tartally et al. (2007) also documented *R. wasmannii* on wingless

Table 2 Infection prevalence of *Rickia wasmannii* in associates found in infected *Myrmica* nests. Numbers in parentheses indicate total individuals; the preceding number shows how many were infected. Asterisks (*) indicate obligate myrmecophilous species

Myrmica sabuleti			*Myrmica scabrinodis*		
Myr_WE_008	Acari		Myr_WE_003	Acari	
	Acariformes – hypopus stage	0 (219)		Acariformes – hypopus stage	0 (1)
	Acariformes – other	0 (369)		Acariformes – Oribatida	0 (73)
	Mesostigmata	0 (9)		Acariformes – other	0 (283)
	Araneae	0 (1)		Mesostigmata	0 (48)
	Insecta			Collembola	
	Coleoptera	0 (1)		*Cyphoderus albinus**	0 (3)
	Myriapoda			Other	0 (4)
	Diplopoda	0 (1)		Insecta	
	Isopoda	0 (3)		Hemiptera	0 (6)
				Thysanoptera	0 (6)
Myr_WE_021	Acari			Nematoda	0 (2)
	Acariformes – hypopus stage	**1** (1)			
	Acariformes – Oribatida	0 (9)	Myr_WE_038	Acari	
	Acariformes – other	0 (3)		Acariformes – hypopus stage	**1** (3)
	Mesostigmata – Uropodina	**16** (18)		Acariformes – Oribatida	0 (20)
	Mesostigmata – Laelapidae	**2** (2)		Acariformes – other	0 (6)
	Araneae	0 (1)		Mesostigmata – Uropodina	0 (2)
	Collembola			Mesostigmata – other	0 (4)
	*Cyphoderus albinus**	0 (8)		Araneae	0 (2)
	Insecta			Myriapoda	
	Hymenoptera	0 (1)		Chilopoda	0 (7)
	Myriapoda			Diplopoda	0 (9)
	Chilopoda	0 (3)		Collembola	
	Diplopoda	0 (2)		*Cyphoderus albinus**	0 (12)
	Isopoda			Other	0 (2)
	*Platyarthrus hoffmannseggii**	0 (2)		Insecta	
	Other	0 (6)		Hymenoptera – Formicidae	0 (21)
	Annelida	0 (1)		Isopoda	
	Nematoda	0 (1)		*Platyarthrus hoffmannseggii**	0 (6)
				Other	0 (8)
Myr_WE_040	Acari			Annelida	0 (1)
	Acariformes – hypopus stage	0 (1)		Nematoda	0 (1)
	Acariformes – Oribatida	0 (18)			
	Acariformes – other	0 (5)	Myr_WE_039	Acari	
	Mesostigmata – Uropodina	0 (3)		Acariformes – other	0 (481)
	Mesostigmata - other	0 (9)		Mesostigmata – Uropodina – *Trichocylliba comata**	**1** (2)
	Collembola			Mesostigmata – other	0 (84)
	*Cyphoderus albinus**	0 (5)		Araneae	0 (3)
	Other	0 (1)		Myriapoda	
	Myriapoda			Chilopoda	0 (11)
	Chilopoda	0 (1)		Diplopoda	0 (11)
	Diplopoda	0 (1)		Symphyla	0 (1)
	Nematoda	0 (1)		Collembola	
				*Cyphoderus albinus**	0 (5)
Myr_WE_041	Acari			Other	0 (13)
	Acariformes – hypopus stage	**3** (36)		Insecta	
	Acariformes – Oribatida	0 (16)		Coleoptera – *Lomechusa emarginata**	**1** (1)
	Acariformes – other	0 (200)		Coleoptera – other	0 (5)
	Mesostigmata	0 (26)		Hymenoptera – Formicidae	0 (17)

Table 2 (continued)

Myrmica sabuleti		*Myrmica scabrinodis*	
Myriapoda		Hemiptera	0 (16)
Chilopoda	0 (1)	Thysanoptera	0 (5)
Diplopoda	0 (4)	Isopoda	
Insecta		*Platyarthrus hoffmannseggii**	0 (30)
Hymenoptera – Formicidae	0 (1)	Other	0 (8)
Diptera	0 (1)	Annelida	0 (13)
Isopoda		Nematoda	0 (10)
*Platyarthrus hoffmannseggii**	0 (3)		
other	0 (7)		
Annelida	0 (3)		

Numbers in parentheses indicate total individuals; the preceding number shows how many were infected. Asterisks (*) indicate obligate myrmecophilous species

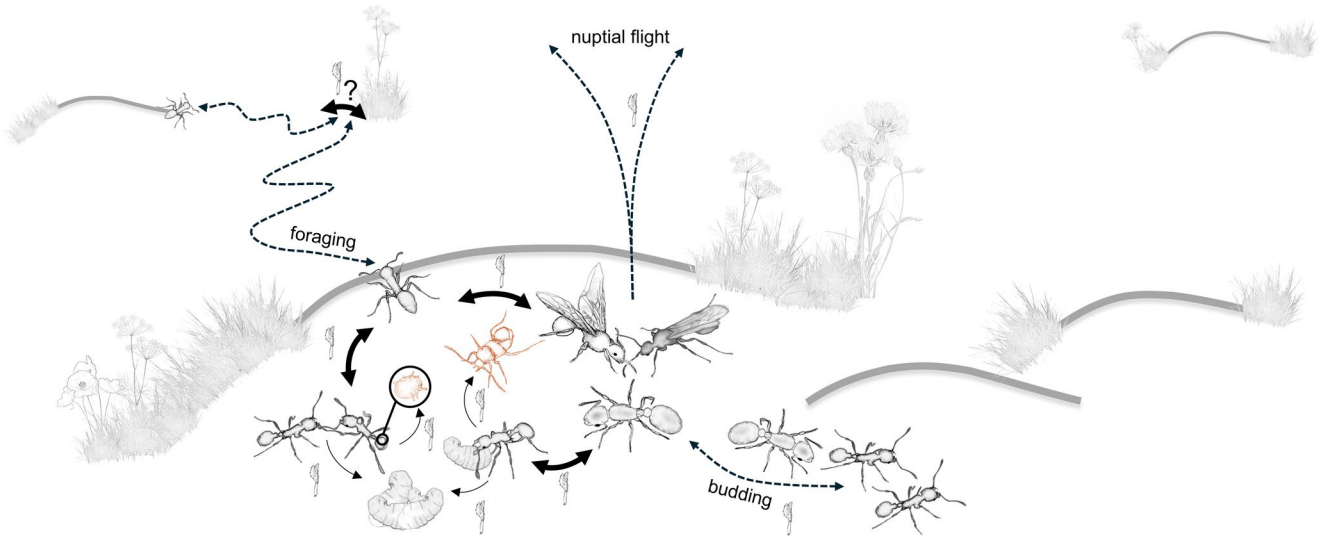

Fig. 3 Hypothesized transmission pathways of *Rickia wasmannii* in the *Myrmica* nest microcosm. The fungus is transmitted among workers and between workers and wingless (reproductive) queens within the nest. Winged sexuals can also become infected when they are present. Larvae and arthropod associates (orange colored) may become infected in the nest, but these hosts are likely to represent dead ends. Transmission between colonies may occur via foraging workers that come into contact with workers from other colonies, for example at shared feeding sites. Whether infection can occur indirectly via spores deposited on the substrate and subsequently contacted by a forager of another colony remains unclear. Winged (virgin) queens and males may disperse the fungus over larger distance during and after nuptial flights, while colony budding may promote transmission over short distances

(reproductive) queens, though they did not detect thalli on winged queens or males. Notably, our study found that both wingless (reproductive) and winged (virgin) queens may be heavily infected with *Rickia* thalli, and that winged males may also carry thalli. To our knowledge, this is the first report of *R. wasmannii* infection on winged ant sexuals. Males and winged queens are only temporarily present in *Myrmica* nests (Seifert 2007) and they were in our study mainly encountered from July to August. This explains why *Rickia* was detected in these castes in only one or two colonies, as most of the seven infected colonies were collected outside this period, when winged sexuals were probably absent. Although we collected few colonies with infected winged sexuals, all sexuals present were infected, suggesting that sexuals may act as a significant reservoir and hub for fungal transmission. As winged sexuals are a major dispersal stage for ant colonies, these findings imply potential, previously unreported transmission pathways for the fungus outside the nest (Fig. 3). It is possible that the fungus hitchhikes on infected sexuals, spreading to new nest sites either with infected winged queens or through males transmitting the fungus to queens. Although *Myrmica* sexuals are expected to differ slightly from workers in cuticle structure (e.g., cuticle thickness) and in cuticular hydrocarbon profiles (Peeters and Ito 2015; Sprenger and Menzel 2020), these differences do not appear to prevent fungal transmission. The lack of records on sexuals is likely due to the fact that winged sexuals inhabit the nest only briefly during the reproductive season and are therefore often overlooked as potential hosts. Another potential transmission route is via

budding of the colony, whereby one or more queens establish a new nest nearby with a group of workers. *Myrmica* colonies are often polygynous and may form these polydomous networks through colony splitting (Radchenko and Elmes 2010). The splitting of an infected mother colony is therefore likely to facilitate short-distance spread of the infection (Fig. 3).

We observed a single mature *Rickia* thallus on a larva (Fig. 2D), whereas a previous report only mentioned immature thalli on *Myrmica* larvae (Baumgartner 1934). Larvae undergo multiple ecdysis events, shedding their cuticles, and the attached thalli, after every molt (three molts over a span of about 30 days). This observation raises the possibility that *Rickia* may reach maturity faster than the 12–15 days previously reported by Baumgartner (1934), although this remains highly speculative given it is based on a single thallus. In addition, larval development and time between different molts may be prolonged due to infection, potentially allowing thalli more time to mature. The findings on larval infections could significantly deepen our understanding of the impact of *R. wasmannii*. Csősz et al. (2021) explained a detected decrease in larval body size following *Rickia* infection by the workers through an indirect mechanism. They hypothesized that the fungus induces stress, causing workers to spend less time with the larvae, which leads to malnutrition. However, *R. wasmannii* infection of the larva cuticle may directly affect larval growth. Importantly, the parasite prevalence and density on larvae (max. two thalli) were much lower than in workers or sexuals. Only one of the twenty detected thalli found on larvae was mature, likely due to the challenges the fungus faces in maturing on the larval cuticle. The absence of functional perithecia in most thalli suggests these infections are mostly a reproductive dead-end, as the thalli may not have enough time to fully develop during the larval instars. Although ant larvae are often closely cared for by multiple workers in a colony and might thus act as a vector spreading infection throughout a colony, our observations suggest that larvae likely do not play a significant role in the transmission dynamics of *Rickia* within and among nests (Fig. 3).

Myrmica ant nests attract a diverse community of arthropod associates that live strictly within them (Parmentier et al. 2020; Witek et al. 2014). We found that ascospores of *R. wasmannii* can develop into thalli on the cuticle of these associates or myrmecophiles, as observed in 24 mites and the rove beetle *Lomechusa emarginata*. These infected associates were found across four of the seven nests of infected *Myrmica* colonies. The number and spread of infections in associated mites may be underestimated. In the three other nests with infected *Myrmica* colonies, we observed individuals that carried no visible thalli, but instead displayed black dots, which likely represent the blackened foot cells of *Rickia* thalli. Our results suggest that transmission to ant associates in nests with infected *Myrmica* colonies can occur (Fig. 3). However, the prevalence of the infected associates (1.2%) is much lower than this of the *Myrmica* workers (100%) and sexuals (100%) in the infected colonies. In a previous report by Pfliegler et al. (2016), immature *R. wasmannii* thalli on *Myrmica* associates were found on six Acaridae mites and one *Microdon* fly larvae with immature thalli. In accordance with that study, thalli on the arthropod associates were all immature. This suggests that these alternative hosts might be a dead-end: they only carry thalli that remain sexually immature.

Apart from unknown physiological and mechanical (structure of the cuticle) barriers that block or hamper the establishment and growth of the fungus on non-*Myrmica* species, the interaction rate with the colony members can explain the lower prevalence. *Myrmica* ants constantly interact (grooming, exchanging food, rubbing) with other members, facilitating direct contact transmission of the fungus across the colony. Ant associates also move among colony members in the nest, enabling potential direct transmission of *Rickia* spores by contact with the host. They also touch the nest's floor and walls, which may act as a reservoir for spores, although indirect transmission is generally considered to be far less important than direct transmission (De Kesel 1995). However, associates have less physical contact with *Myrmica* nestmates than *Myrmica* nestmates have with one another. They also vary greatly in the level of interaction with the host, ranging from unspecialized species that mostly avoid direct contact with the host and remain on the nest periphery, to specialized species those treated as nest mates, entering the dense brood chambers and engaging in frequent grooming and mouth-to-mouth feeding interactions with the host (Hölldobler and Kwapich 2022; Parmentier 2020). Additionally, some species are phoretic or ectoparasitic and therefore remain in constant close contact with their hosts. Interestingly, phoretic mites have been suggested as potential vectors of Laboulbeniales, transmitting these fungi to their arthropod hosts, such as ants (Santamaria and Espadaler 2015) and beetles (Seeman and Nahrung 2000). In our observations, infected associates were limited to those with either a phoretic stage attached to the host, ectoparasitic behavior or a strong integration into the colony. The infected mites included phoretic Astigmata and two Laelapidae individuals, a group capable of phoresy (Walter and Moser 2010). Other infected mites were members of Uropodina, an ectoparasitic group (Klompen and Campbell 2025), with one specimen identified as *Trichocylliba comata*.

The *Lomechusa* beetle exhibits one of the most advanced repertoires of behavior among myrmecophilous rove beetles. It is fed, groomed, and transported throughout the

nest without provoking aggression. The *Microdon* larva and mites previously observed with thalli of *R. wasmannii* (Pfliegler et al. 2016) are also examples of highly integrated associates and phoretic stages, respectively. Obligate myrmecophiles such as the isopod *Platyarthrus hoffmannseggii* and the springtail *Cyphoderus albinus*; that we frequently found with severely infected *Myrmica* colonies, showed no signs of thalli. In contrast to *Lomechusa* and *Microdon*, they interact poorly with the host. Infections in facultative associates were not recorded, including the Oribatida mites which scavenge in the organic material but hardly interact with the host. Our observations, together with those of Plieger et al. (2016), are consistent with the hypothesis that closer integration with the host may facilitate infection, although this interpretation is limited by the small number of infected individuals observed. Given the lower fungal prevalence and thallus density on myrmecophiles compared to workers, the transmission of the infection across colonies is probably much less common via dispersing myrmecophiles and mites in particular (Parmentier et al. 2021; Fig. 3), even though it was suggested that other *Rickia* species have been transmitted using mites as vectors (Seeman and Nahrung 2000; Santamaria and Espadaler 2015).

Previous work has shown that *Rickia* infection in *Myrmica* workers exhibits seasonal variation, peaking after winter and declining through summer and autumn (Haelewaters et al. 2015b). In our study, however, we did not detect clear seasonal patterns in worker infection: the seven colonies with infected workers were collected between May and October, and workers were fully infected in all colonies, including the one collected in October. Infected larvae were observed in two colonies collected during autumn, and infected myrmecophiles were collected from July to October, but further data are needed to clarify potential seasonality of infection in these groups. The seasonal infection variation we observed in the microcosm appears to be largely driven by the short-lived presence of winged castes in summer, which can only become infected during this short period.

This study broadens the known host range of *R. wasmannii* in natural conditions, showing that the fungus is not restricted to the worker caste of *Myrmica* ants. Instead, it appears capable of infecting other *Myrmica* castes, including larvae, as well as arthropod associates in the nest environment. For the fungus, however, the importance of alternative arthropod hosts may be limited and probably represent dead-ends, given the low prevalence and density of the fungus, as well as the absence of mature thalli on these hosts.

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Author contributions Thomas Parmentier: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing—Original Draft, Writing—Review & Editing, and Resources. Jelter Bekaert: Methodology, Investigation, and Writing—Original Draft. Lars Westerlinck: Methodology, Investigation, Writing—Original Draft, and Visualization. Koen Martens: Methodology, Investigation, and Visualization. Jenő Kontschán: Investigation. André De Kesel: Investigation, Visualization, and Writing—Review & Editing. Danny Haelewaters: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing—Original Draft, Writing—Review & Editing, Supervision, and Resources.

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Data availability The raw data are available in the manuscript in Table 1 and 2.

Declarations

Conflict of interest The authors declare that they have no financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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