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1 **Entomopathogenic *Rickia wasmannii* fungal infection generated colony-level stress effects**
2 **cause size decline in *Myrmica* ants**

3

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19

20 **Abstract**

21 Parasitism-generated negative effects on ant societies are multifaceted, implying individual and
22 colony-level responses. Though laboratory based evidence shows that the sublethal fungus
23 *Rickia wasmannii* is responsible for physiological and behavioral responses that may negatively
24 affect individual workers' resilience and life expectancy in *Myrmica* ant workers, colony-level
25 stress response to this parasite is largely unknown. Here, we focus on understanding of a long-
26 term, colony-level effect of *Rickia* infection on *Myrmica scabrinodis* ant populations by
27 tracking trait size-based changes. We collected worker specimens from infected and uninfected
28 colonies from the same population in order to: (i) compare body size in response to parasitism,
29 (ii) assess the extent to which possible changes in size are associated with the severity of

30 infection, and (iii) investigate shifts in body size in response to infection over time by testing
31 correlation of workers' ages and sizes. We found that workers from infected colonies were
32 significantly smaller than their healthy congeners, but neither infection level nor the age of the
33 workers showed significant correlation with the size in infected colonies. Decreasing body sizes
34 in infected colonies can be ascribed to workers' mediated stress toward developing larvae,
35 which are unable to attain the average body size before they pupate.

36

37

38 **Introduction**

39 Ants (Hymenoptera: Formicidae), the most widespread social organisms on Earth, attract an
40 amazing diversity of parasitic organisms, such as viruses ^[1], bacteria ^[2], fungi ^[3,4], and an array
41 of uni- and multicellular animal organisms ^[5,6]. Many of these parasites cause lethal diseases
42 ^[7], but most are sublethal, i.e. they do not necessarily pose an imminent danger, though they are
43 assumed to have detrimental effects on the quality of the hosts' lives ^[4,8].

44 Entomopathogenic fungus, *Rickia wasmannii* Cavara, 1899 (Ascomycota: Laboulbeniales) is a
45 typical sublethal parasite of several *Myrmica* (Hymenoptera: Formicidae) species which has
46 long been believed to have no detrimental effect on its host individuals ^[9,10,11]. For a century,
47 very little was known about this parasitic organism, and only scant information was available
48 on their distribution and host specificity ^[4,12,13]. The effect of the fungus on the physiology of
49 its host species was largely unknown. In recent years, modern research has shed light on the
50 real nature of this widespread organism and has shown that it has negative effects on individual
51 host ants. Based on previous studies on the physiological response of infected workers of the
52 main host *Myrmica scabrinodis* Nylander, 1846, infected workers show higher mortality under
53 laboratory conditions ^[14,15]. Infected *M. scabrinodis* workers were also shown to exhibit
54 improved sanitary behavior ^[14] and a decreased level of intraspecific aggressivity ^[16,17] which
55 may be disadvantageous for the infected colonies in competitive interactions.

56 Although *R. wasmannii* is known to elicit different detrimental physiological responses in
57 individual workers, colonies seem to resist and compensate for the negative effects of the
58 infection; they contain queens, rear larvae and pupae, and all age-classes of workers are present
59 (see Csata et al. ^[17]). This may be due to the fact that it is often challenging to study colony-
60 level effects of an infection under in situ conditions, particularly when complex environmental
61 parameters and multifactorial relationships with a number of other organisms ^[8,15,18] must be

62 taken into consideration. This may be one of the reasons why a colony-level stress effect of
63 *Rickia* fungal parasites has never been the subject of focused research.

64 We propose that the colony-level negative effect of the infection is detectable through the
65 decline in size of colony workers. Here, we hypothesize that the infection has detrimental
66 effects on the fitness of brood caring workers, making them unable to engage in foraging
67 activity and feeding and grooming behavior toward their larvae to the necessary extent, and the
68 declining larval growth rate ultimately leads to a decrease in the size of the next generation of
69 workers. The size of the first workers infected is not affected, but *R. wasmannii* infection is
70 transferred over generations, causing a long-term chronic, life-long infection in a colony, which
71 is known to expand year by year in the nest with some level of seasonal fluctuation ^[19]. The
72 detrimental colony-level effect of the infection accumulated over time can be measured in
73 workers' sizes. We hypothesized that if *R. wasmannii* infection is widespread in an ant colony,
74 it will detrimentally affect the size of workers groomed by infected sisters owing to the parental
75 colony's reduced fitness. To test this hypothesis, we compared the sizes of randomly sampled
76 workers from infected and uninfected colonies.

77 How can we rule out the possibility that the size decline is caused not by direct larval infection
78 but by mediated colony-level stress among workers? *Rickia wasmannii* fungus is found to grow
79 on imago ^[14,20] and is not known to infect ant larvae ^[11,21] but in order to make sure that a
80 possible size decline is ascribed to stress mediated by nursing workers and is not caused by
81 direct larval infections, trait scaling patterns were also observed. The background of this
82 approach is that infections in the larval stage often cause scrambled trait combinations in adults
83 ^[22] via altered static trait allometries. If significant shifts in trait scaling were detected in
84 infected colonies, parasitism generated stress might not only be mediated by infected workers.

85 We also tested whether the extent of infection (i.e., number of thalli on the cuticular surface of
86 the ants) and the workers' ages affect the detected changes in imaginal size. Therefore, we
87 registered both infection level and estimated age of the workers in the colony.

88 Understanding the colony-level effect of *R. wasmannii* infection on *Myrmica* colonies in a
89 natural environment would foster a better understanding of the dynamics of this
90 enthomopathogenic fungus and the host-parasite system. This issue has interesting
91 implications, because *Myrmica* ants are a known host species of a guild of ant guests and
92 socially parasitic organisms which live together with their colonies ^[8,23]. This network is very

93 sensitive, and every single component of this very complex system might have a regulatory role
94 of its own.

95

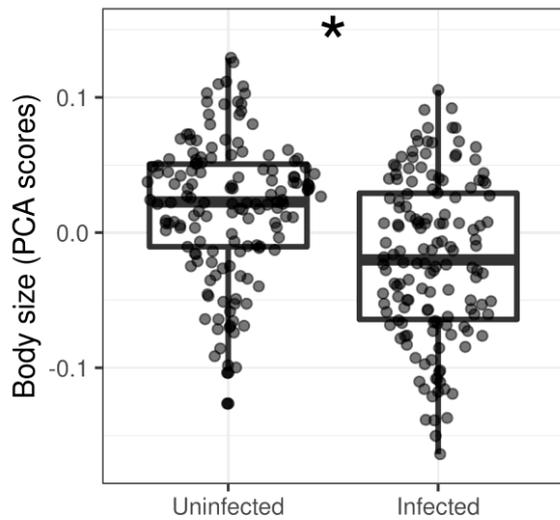
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97 **Results**

98 A total of 300 workers from 30 colonies were measured (15 colonies of each class). The
99 calculated Intra Class Correlation between 16 pairs of repeated measures was very high ($R =$
100 0.980 to 1.0), i.e. measurement error was negligible. Body size was significantly decreased in
101 infected colonies in comparison to uninfected ones ($\beta = -0.038$, $SE = 0.014$, $t = -2.66$, $P = 0.013$,
102 Fig. 1). We found no significant effect of age ($\beta = -0.003$, $SE = 0.005$, $t = -0.65$, $P = 0.514$) or
103 thalli number ($\beta = 0.0004$, $SE = 0.006$, $t = 0.06$, $P = 0.950$, Fig. 2) on body size among infected
104 colonies. Variance inflation factor was 1.025 for both independent variables, suggesting no
105 substantial multicollinearity. Nevertheless, based on the Poisson GLMM, age had a significant
106 positive effect on thalli number ($\beta = 0.405$, $SE = 0.011$, $t = 35.71$, $P < 0.001$, Fig. 3).
107 Furthermore, we found no significant difference between trait correlation matrices of uninfected
108 and infected colonies, indicating that they exhibit the same allometries ($X^2 = 1.313$, $P = 0.999$,
109 Fig. 4).

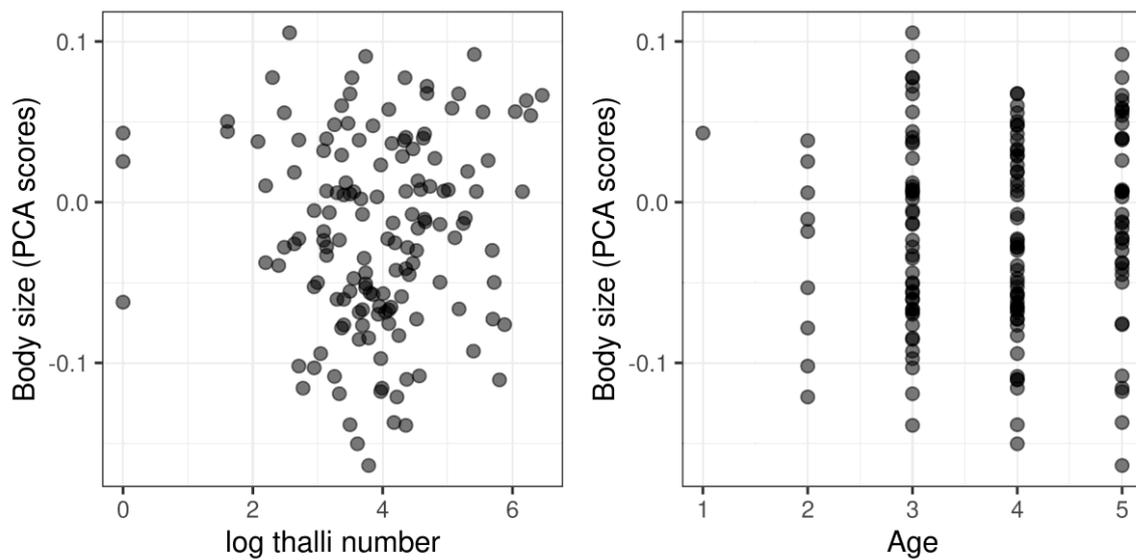
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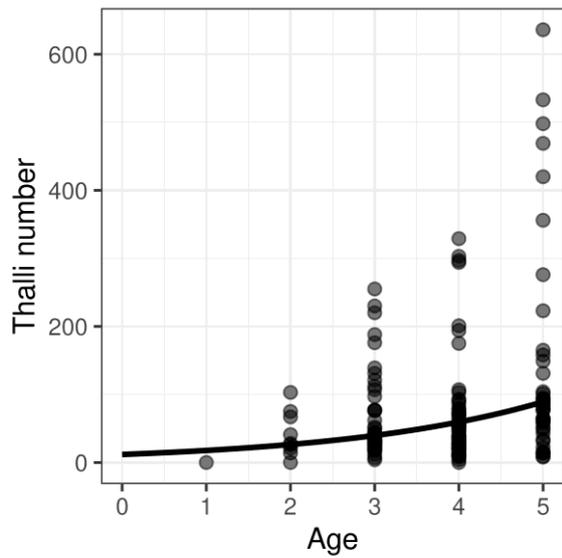
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 113 Fig. 1. Distribution of PCA axis values representing body size in uninfected and infected
 114 colonies. Asterisk marks statistically significant difference.

115



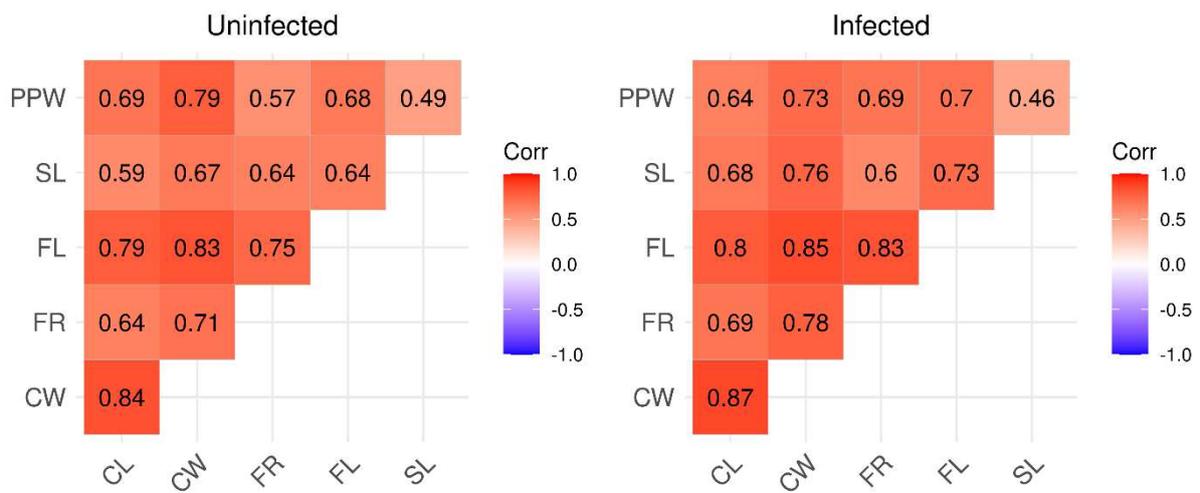
116
 117 Fig. 2. Visualization of how log-transformed thalli number (left panel) and age (right panel) are
 118 associated with body size; based on our LMM neither of them had significant effect on the
 119 workers' size.

120



121
 122 Fig. 3. Association of age and thalli number of workers among infected colonies. The solid line
 123 represents the association predicted on the Poisson GLMM.

124



125
 126 Fig. 4. Correlograms visualizing the trait associations of measured body size variables in
 127 uninfected (left panel) and infected (right panel) colonies.

128

129 **Discussion**

130 Our results show that *R. wasmannii* infected *M. scabrinodis* workers are significantly smaller
131 in a native environment than uninfected ones. This decline in body size in the infected
132 population is consistent across all observed characters. It is important to note that we did not
133 detect shifts in allometries in the characters measured. The significant decrease in size took
134 place synchronously. The lack of shifts in static trait allometries indicates that the size decline
135 in the next generation of workers is not ascribed to physiological stress due to direct larval
136 infections but to a colony-level stress effect caused by widespread infection of *R. wasmannii*
137 fungus across the colonies' adults. Discrete factors, such as parasitism, are known to modify
138 larval development through altered physiological processes, and these developmental
139 perturbations leave their mark on the allometry of some traits of subsequent adults ^[22], but no
140 traces of such parasitism are detected in natural *Myrmica* populations. We suppose that a
141 colony-level stress in response to *R. wasmannii* infection is mediated toward larvae by nursing
142 workers, resulting in significantly smaller static trait size among the next generation of their
143 nest mates.

144 Earlier, laboratory based findings and field studies have shown that this fungal infection does
145 not attack *Myrmica* larvae ^[11,21,24,25], but is known to cause an array of detrimental changes in
146 physiology in individual ant workers, such as a decrease in life expectancy, particularly in cases
147 of water and food deprivation ^[15], increased sanitary behavior ^[14], and a reduced ability to fight
148 against competitors ^[16,17]. Yet, despite the number of negative effects detected under laboratory
149 conditions, infected colonies in the field have been thought to function *relatively well, as they*
150 *contain queens, larvae and pupae, and all age-classes of workers* (see Csata et al. ^[17]).

151 Our research is the first undertaking that provides quantitative evidence concerning a decline
152 in worker size in infected nests, dispelling the previous concept concerning the seeming health
153 of infected colonies and revealing that chronic *R. wasmannii* infection has a long,
154 intergenerational, detrimental colony-level impact in the natural environment.

155 Infected colonies can somehow manage to compensate for the negative effects of *R. wasmannii*
156 infection (colonies operate with queens, larvae and pupae and they have the capacity to produce
157 sexual forms and maintain their populations), but certain functions are clearly impaired, which
158 is reflected in the decrease in body size among the next generation of workers.

159 The background of the workers' mediated colony-level stress as a consequence of parasitism is
160 not entirely clear. Similarly to other members of the order Laboulbeniales, *R. wasmannii* does

161 not penetrate the cuticle of the host, so the most likely hypothesis concerning the feeding of the
162 parasitic fungus is that it absorbs the necessary nutrients from the workers' cuticle surface or
163 directly from the environment ^[25]. Ants have numerous exocrine glands, the secretions of which
164 are spread on the cuticle surface by self-grooming and allogrooming. This may be confirmed
165 by the fact that infected *M. scabrinodis* workers show increased sanitary behavior ^[14]. This
166 behavior has been observed in the invasive garden ant (*Lasius neglectus* van Loon boomsma et
167 Andrásfalvy, 1990) infected by *Laboulbenia formicarum* Thaxt. ^[26], where fungus also was not
168 found to penetrate the cuticle of its hosts ^[25]. This increased sanitary behavior could mean that
169 ant workers have less time and energy to care for and feed the brood, which could be another
170 explanation for the decline in body size in subsequent generations in infected colonies.

171 This might be explained by the discrete, i.e., presence-absence type of stress caused by *Rickia*
172 infections on colonies, though we do not find this explanation the most persuasive one. The
173 most plausible reason might be lurking in the dynamics and phenology of the infection, namely
174 the level of infection might not correlate with the number of visible thalli on the worker
175 individuals' cuticular surface. We also know that the intensity of infection varies throughout
176 the year, and the peak number of thalli takes place in late autumn ^[19]. This fluctuation, combined
177 with the emergence of smaller workers from the overwintering larvae of *Myrmica* ants ^[27],
178 makes it difficult to map the exact processes.

179 Although only negative effects of the fungus are currently known, we speculate that the
180 increased sanitary behavior may be advantageous against other parasites, and *R. wasmannii*
181 may also have unknown positive effects on its hosts, similar to the *L. formicarum* ^[14,26].

182 We also speculate that the decline in size among workers in infected *Myrmica* ant colonies is
183 part of the colony-level strategy to minimize the adverse effects of the infection in order to
184 mitigate increased mortality and maintain the ability to produce sexuals. From an evolutionary
185 biological perspective, whether a *R. wasmannii* infection impacts the fitness of *M. scabrinodis*
186 colonies is an important question. We do not know whether infected colonies produce smaller
187 or perhaps fewer sexuals or this ultimate function is not impaired. This topic merits further
188 investigation.

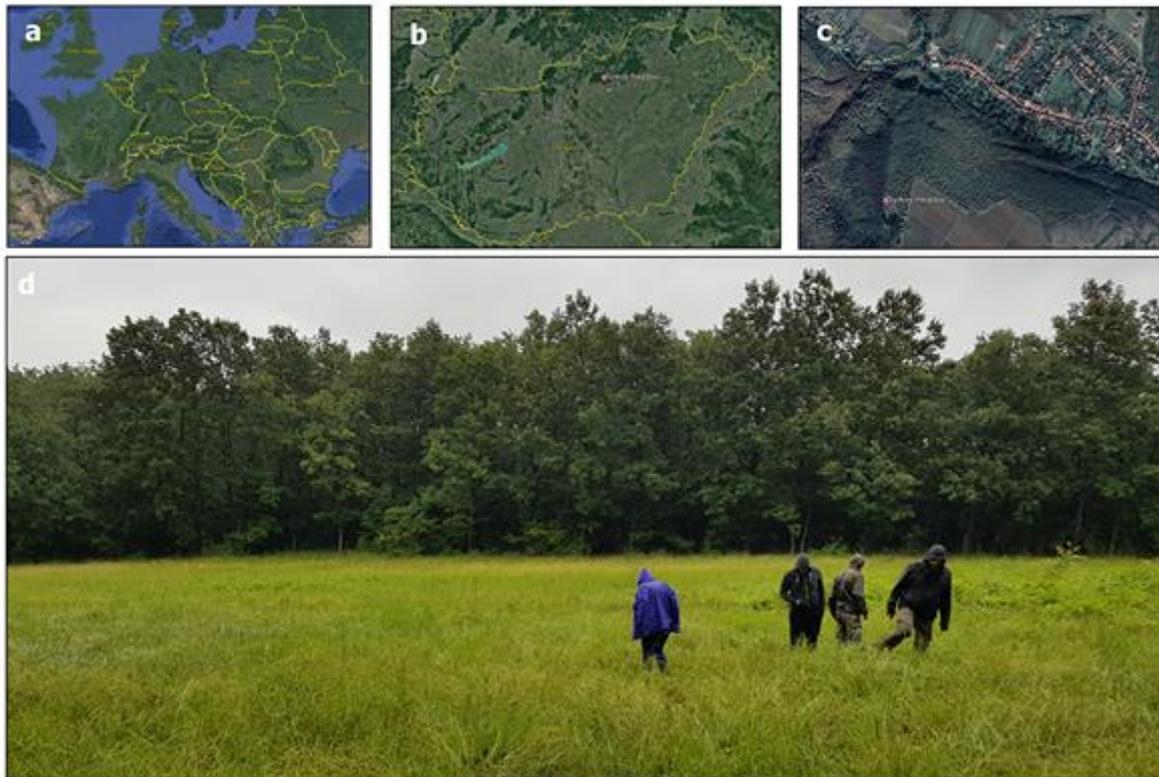
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190

191 **Materials and Methods**

192 *Study site*

193 Material for the present study was collected from a relatively small, one-acre fragment of a
194 natural marshland surrounded by deciduous, oak-dominated forest near Gyöngyös, Sár-hegy:
195 Gyilkos-rét (23.06.2016; 47.811206, 19.988027; 320 m a.s.l) in Hungary (Fig. 5). This area is
196 part of the Bükk National Park and has not been disturbed or extensively cultivated over the
197 course of the past two decades.



198

199 Fig. 5. Field site where *M. scabrinodis* workers for our study were collected. Field site is located
200 in Europe (a), Hungary (b) near Pálosvörösmart (c). Wet meadow (Gyilkos-rét) surrounded by
201 deciduous forest (d).

202

203 *Sampling*

204 Infected (Fig. 6) and uninfected *M. scabrinodis* colonies were found via hand searching in the
205 grassland by FB and AT. Sampling activities were concentrated in a short, one-day period of
206 time (23.06.2016) because *R. wasmannii* infection is known to show seasonal fluctuation ^[19].
207 Altogether 30 *M. scabrinodis* colonies were used as samples, and 15 uninfected and 15 infected
208 specimens were taken from each colony. The nests were carefully opened and the presence of

209 *R. wasmannii* on the workers' body surfaces was checked in every colony with the use of a 40x
210 magnifying glass in the field. After inspection, a small fragment of colony workers was
211 collected from each nest. The sampled workers were stored in 1.5 mL Eppendorf tubes with
212 67.5% EtOH until observation in the laboratory.

213

214 *Thallus number on the workers' cuticular surfaces*

215 Altogether, 225 *M. scabrinodis* workers (15 individuals from each infected colony) were
216 screened (by FB) for fungal thalli (Fig. 6). All fungal thalli on the whole ant body were counted
217 using a Leica MZ125 stereomicroscope at $\times 10$ -160 magnification as described by Báthori et al.
218 [20]. After the fungal thalli had been counted, colony infection level was calculated based on
219 arithmetic mean thalli number of 15 randomly sampled workers from each colony.

220



221

222 Fig. 6. Habitus image of an infected *Myrmica scabrinodis* worker. *Rickia wasmannii* thalli
223 cover the head, mesosoma, and, to a lesser extent, the appendages. Photo: Zsolt Újvári.

224

225 *Estimating worker age*

226 As was done by Báthori et al. ^[20], all infected *M. scabrinodis* workers screened for fungal thalli
227 were separated into different age groups. Based on the degree of cuticular pigmentation, five
228 different age categories were described by Cammaerts-Tricot ^[28]. The infected individuals were
229 classified into categories according to cuticle coloration from younger to older (1-5). The highly
230 pigmented oldest workers were given the highest numbers.

231

232 *Morphometric character recording*

233 The measured morphometric characters are defined as in ^[22]. The measurements of altogether
234 300 *M. scabrinodis* workers (10 from each colony) were made with an ocular micrometer using
235 a Leica MZ125 stereomicroscope at a magnification of $\times 50$ for CL, CW and FR, $\times 100$ for FL,
236 SL and PPW (all measurements were recorded in μm). All measurements were made by FB.
237 Measured characters are defined in Table 1. Raw data are available in Supplementary table 1.

238

239 *Measurement error assessment*

240 All measurements are subject to error, therefore repeatability, i.e., the degree of agreement
241 between pairs of observations made on the same measurand under the same conditions, i.e.
242 made by the same observer, using the same microscope, following the same measurement
243 protocol as defined in Csósz et al. ^[29], was tested before the statistic framework was created.
244 The repeatability of the recorded size parameters was assessed via Intraclass Correlation
245 Coefficients (ICC) on repeated measurements of 16 ant specimens using Package ICC (Wolak
246 et al. 2012). ICC scores are given for each characteristic in Table 1.

247

Abbr.	Description of traits	ICC
CL	Cephalic length measured from the anterior-most point of clypeal margin to the mid-point of the occipital margin, in full-face view.	0.990 [0.980, 1.000]
CW	Cephalic width measured in full-face view, including compound eyes.	0.997 [0.994, 1.000]

FR	Frons width measured according to the minimum distance between the frontal carinae.	0.995 [0.990, 1.000]
FL	Frontal lobe width measured according to the maximum distance between external borders of the frontal lobes.	0.995 [0.991, 1.000]
SL	Scape length measured from the neck to the distal end of the scape.	0.993 [0.986, 1.000]
PPW	Postpetiole width measured according to the maximum width of the postpetiole in dorsal view.	0.996 [0.991, 1.000]

248

249 Table 1. Verbatim trait definitions for morphometric character recording. Abbreviation (Abbr.),
250 verbatim character definition and intraclass correlation coefficients (ICC) of certain
251 morphometric traits are provided. Upper and lower bounds of ICC scores, separated by a coma,
252 are also given in parentheses.

253

254 *Statistical analysis*

255 All data analyses were performed with R version 4.0.2. ^[30]. To reduce the number of variables
256 on body size we used principal component analysis (PCA) by non-linear iterative partial least
257 squares (NIPALS) with the R-package “nipals” ^[31]. We preferred this method over classical
258 PCA because in a small number of cases (7 in total) size measurements of some body parts for
259 a given ant were not feasible (hence the missing measurements); NIPALS can use data with
260 missing observations, whereas in classical PCA we should have excluded all those specimens
261 for which any measurement was missing. Variables were centered at zero (by subtracting
262 variable mean from each value) and rescaled (by dividing all values by the variable standard
263 deviation) in order to bring them to the same scale. We retained only the first PCA axis (being
264 the only axis with an eigen-value higher than 1), which corresponded to 77% of the total
265 variation in the six body measurement variables and was positively correlated with all variables.

266 To test whether there is a significant difference in body size between infected and uninfected
267 colonies, we used a mixed-effects linear regression model (LMM) with Gaussian error
268 distribution using the R-packages “lme4” and “lmerTest”^[32,33], specifying the abovementioned
269 PCA axis representing body size as the dependent variable and infection as independent factor.
270 To test how the severity of fungal infection and age affected body size, we fitted another LMM
271 (naturally, only using data from infected colonies) with the body size PCA axis as a dependent
272 variable and the log-transformed number of thalli and age as independent variables. Because
273 previously it had been shown that age and thalli number can be correlated, we estimated
274 variance inflation factors to see whether there is substantial multicollinearity between the
275 independent variables used and the “car” package^[34]. In addition, we tested the association
276 between age and thalli number using a Poisson generalized LMM (GLMM), specifying thalli
277 number as a dependent variable. In both LMMs and in the GLMM, colony number was used as
278 a random effect to control for the non-independence of observations from the same colonies.

279 Furthermore, to see whether the infection causes changes in static trait allometries across the
280 measured body size indices, we estimated trait correlations (Pearson’s ρ) separately for
281 uninfected and infected colonies and compared the resulting two correlation matrices^[35].

282

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417

418 **Author contributions**

419 SC and FB contributed to the study conception and design. Data preparation, data collection
420 was performed by FB, LEB. Data analyses were performed by SC and ZR. The first draft of the
421 manuscript was written by SC, FB and AT and all authors commented on previous versions of
422 the manuscript. All authors read and approved the final manuscript.

423

424 **Data availability**

425 Raw data are available from the Supplementary table 1.

426

427 **Additional Information**

428 The authors declare that there is no conflict of financial and non-financial interest.

429

Figures

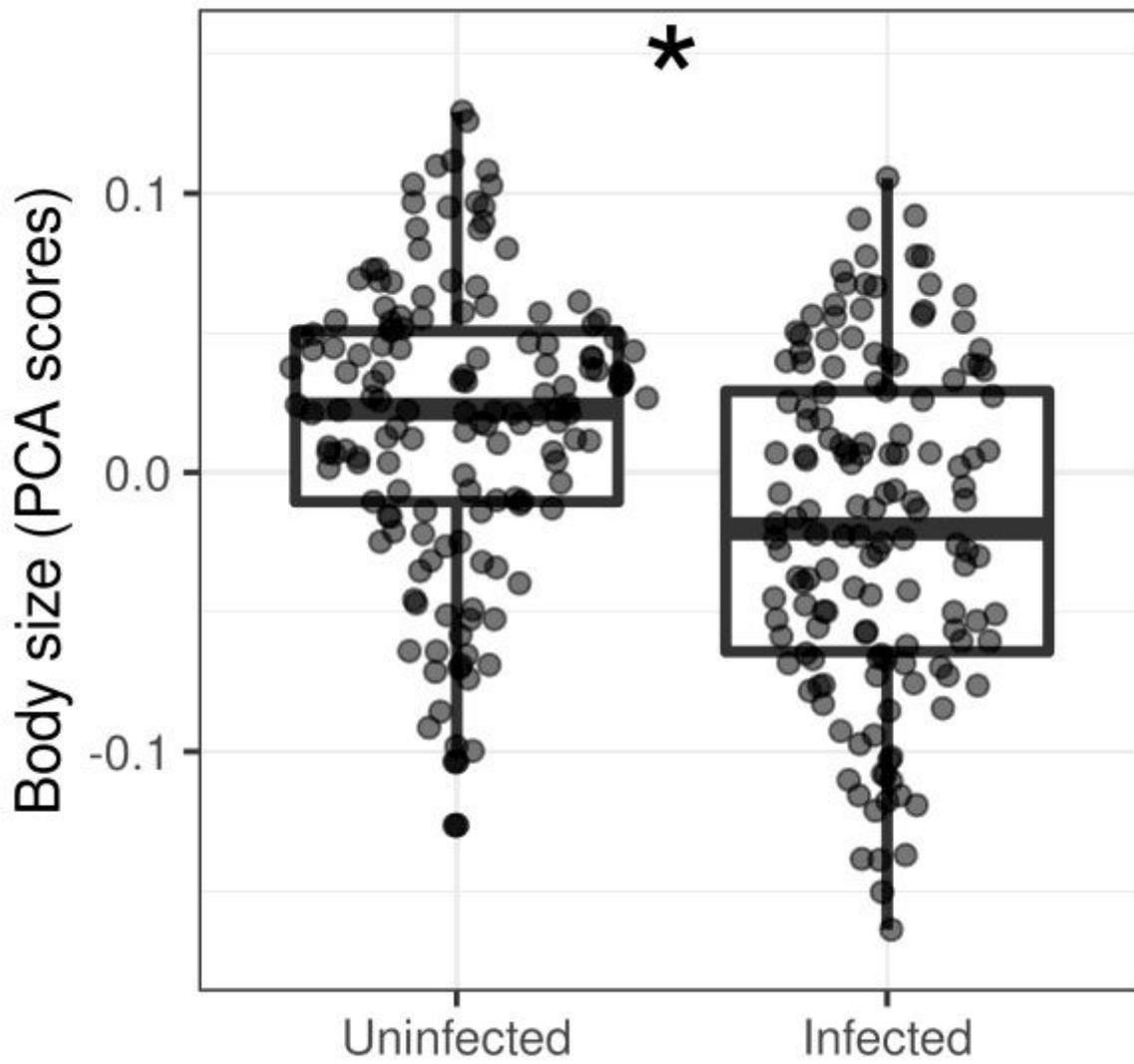


Figure 1

Distribution of PCA axis values representing body size in uninfected and infected colonies. Asterisk marks statistically significant difference.

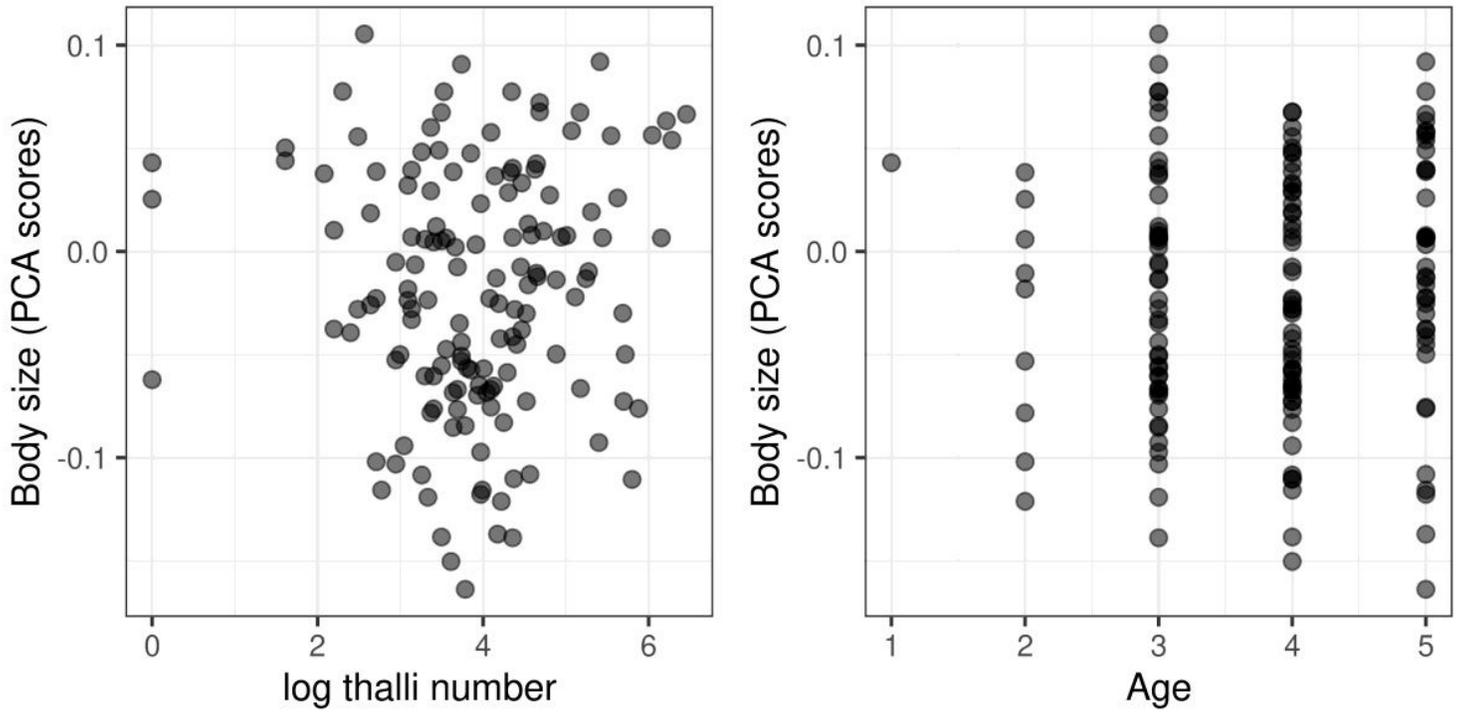


Figure 2

Visualization of how log-transformed thalli number (left panel) and age (right panel) are associated with body size; based on our LMM neither of them had significant effect on the workers' size.

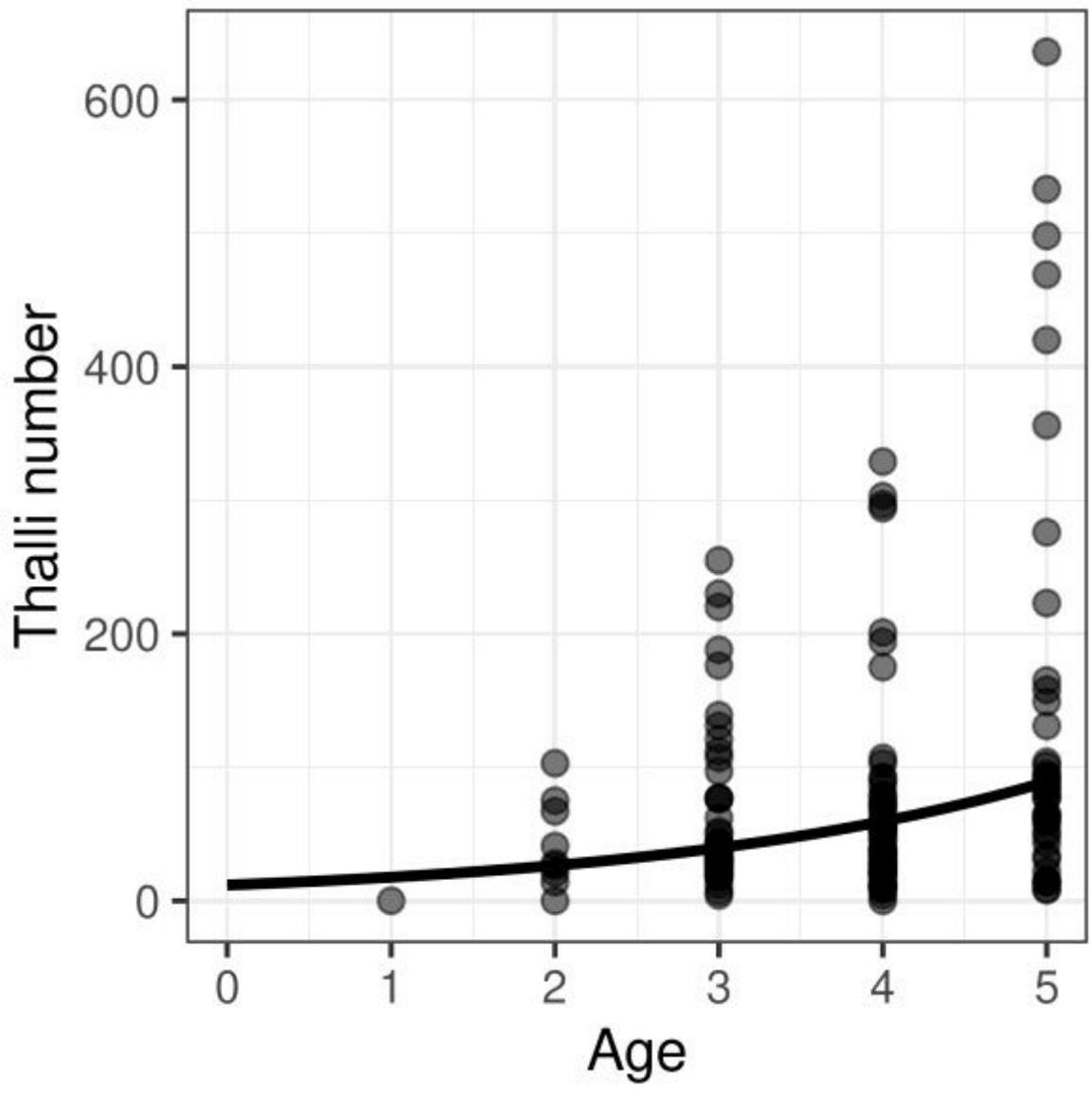


Figure 3

Association of age and thalli number of workers among infected colonies. The solid line represents the association predicted on the Poisson GLMM.

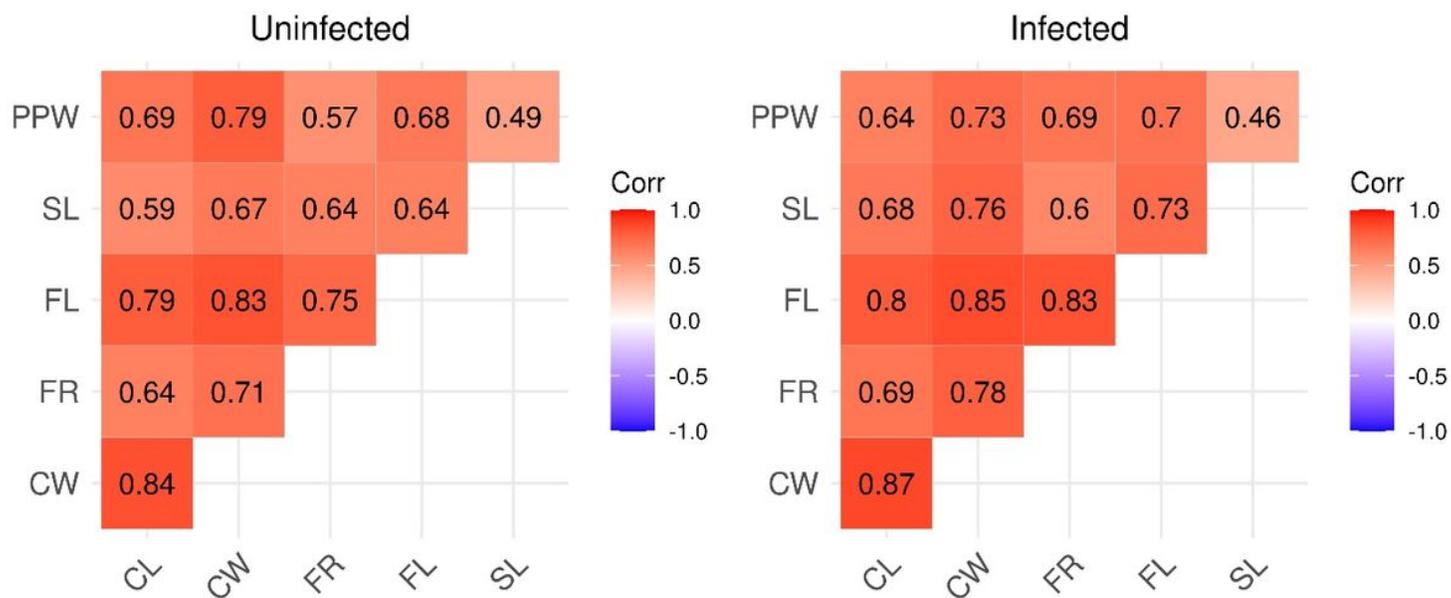


Figure 4

Correlograms visualizing the trait associations of measured body size variables in uninfected (left panel) and infected (right panel) colonies.



Figure 5

Field site where *M. scabrinodis* workers for our study were collected. Field site is located in Europe (a), Hungary (b) near Pálosvörösmart (c). Wet meadow (Gyilkos-rét) surrounded by deciduous forest (d).



Figure 6

Habitus image of an infected *Myrmica scabrinodis* worker. *Rickia wasmannii* thalli cover the head, mesosoma, and, to a lesser extent, the appendages. Photo: Zsolt Újvári.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable1.xlsx](#)