



Non-target effects of *Metarhizium brunneum* (BIPESCO 5/F 52) in soil show that this fungus varies between being compatible with, or moderately harmful to, four predatory arthropods

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1 **Non-target effects of *Metarhizium brunneum* (BIPESCO 5/F 52) in soil show that this**
2 **fungus varies between being compatible with, or moderately harmful to, four**
3 **predatory arthropods.**

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11

12 **ABSTRACT**

13 Biological control with entomopathogenic fungi is a feasible option for regulation of pest insect
14 populations. However, possible effects on beneficial arthropods must be considered. We assessed
15 the non-target effects of the microbial biological control agent *Metarhizium brunneum* (isolate
16 BIPESCO 5/F 52) applied in soil on four different predatory arthropods: the predatory mite
17 *Gaeolaelaps aculeifer* (Canestrini), the predatory bug *Orius majusculus* (Reuter), the rove beetle
18 *Dalotia coriaria* (Kraatz) and the gall midge *Aphidoletes aphidimyza* Rondani. All are widespread and
19 naturally occurring in Europe, they represent different classes of arthropods and different insect
20 orders; furthermore, their life cycles involve different levels of contact with the soil. Adult *G.*
21 *aculeifer*, *O. majusculus*, and *D. coriaria*, and last instar *A. aphidimyza* larvae were exposed to
22 natural soil (control) or natural soil inoculated with *M. brunneum* at a concentration of 5×10^6
23 conidia/g of soil; this represents a worst-case scenario. Mortality, longevity, fecundity and
24 *Metarhizium* outgrowth on dead individuals were assessed for the first three species; for *A.*
25 *aphidimyza*, only mortality (non-emergence rate) and fecundity of emerged females were assessed.
26 The fungal treatment resulted in a significantly higher mortality of *O. majusculus* and *D. coriaria*,
27 96%, and 7.3% respectively, compared with 19%, and 2% for their respective controls. Mortality of
28 *G. aculeifer* was not significantly affected by exposure to the fungus in the soil. Longevity of *O.*
29 *majusculus* and *D. coriaria* was significantly reduced following exposure to the fungus in the soil
30 (log-rank test: $p < 0.0001$, Wilcoxon test $p < 0.0001$ and log-rank test: $p = 0.029$, Wilcoxon test:
31 $p = 0.027$, respectively), while *G. aculeifer* longevity was not affected. Fecundity of *O. majusculus* and
32 *D. coriaria* was negatively affected following exposure to the fungus in the soil, which reduced their
33 oviposition by 20% and 4%, respectively, compared with the control, while *G. aculeifer* fecundity
34 was not affected. *Aphidoletes aphidimyza* larval mortality was higher following exposure to the
35 fungus in the soil (60% dead) than in the control (40% dead) but its fecundity was not statistically
36 significantly affected by treatment. In conclusion, the predatory arthropods studied demonstrated
37 a range of fitness responses to *M. brunneum* exposure in the soil, from no response (*G. aculeifer*),
38 to intermediate (*D. coriaria* and *A. aphidimyza*) and high response (*O. majusculus*). This study
39 demonstrates the relevance of using several fitness parameters and different arthropod species to
40 determine whether a biological control agent should be considered a low-risk substance with
41 respect to non-target effects.

42 **Key words:** Biological control; entomopathogenic fungus; gall midges; predatory mites; rove
43 beetles; predatory bugs.

44 **1. Introduction**

45 *Metarhizium* Sorokin (Ascomycota: Hypocreales) is a genus of entomopathogenic fungus that is
46 often associated with soil ecosystems; it includes species that are commonly used for biological
47 control of numerous insect pests that are economically important in agriculture. The well-known
48 and commercially available strain of *Metarhizium brunneum* Petch, BIPESCO 5/F 52, is highly
49 effective against a number of pests including wireworms (Ansari et al., 2009) and weevils (Nielsen
50 et al., 2006; Klingen et al., 2015). Experimentally, it has shown good establishment and conidial
51 persistence in the field (Pilz et al., 2011), and incremental increases in crop yield have been
52 documented following its use (Kabaluk and Ericsson, 2007).

53 Side-effect studies are essential for registration of microbial biological control products in the
54 E.U. (Sundh and Goettel, 2013). As many species with potential as microbial control agents have a
55 wide host range, non-target effects must be considered critically (Babendreier et al., 2015). For
56 example, inundative application of *Metarhizium* species on to, or into, the soil may have sublethal
57 effects on predatory arthropods that have soil-dwelling phases in their lifecycle (Babendreier et al.,
58 2015).

59 In order to assess non-target effects of soil application of *M. brunneum*, we selected four
60 predatory arthropods that are widespread in Europe and are also commercially available as effective
61 biological control agents in their own right: the mite *Gaeolaelaps aculeifer* (Canestrini) (Acari,
62 Laelapidae), the predatory bug *Orius majusculus* (Reuter) (Hemiptera: Anthocoridae), the rove
63 beetle *Dolania (= Atheta) coriaria* Kraatz (Coleoptera: Staphylinidae) and the gall-midge *Aphidoletes*
64 *aphidimyza* Rondani (Diptera: Cecidomyiidae). *Gaeolaelaps aculeifer* is a mesostigmatic mite from
65 the family Laelapidae; this family is one of the most abundant and species-rich groups of arthropods
66 in the soil (Strong and Halliday, 1994; Navarro-Campos et al., 2012) and has been successfully used
67 for the control of thrips (Navarro-Campos et al. 2012), bulb mites (Amin et al., 2014) and Western
68 corn rootworms (Prischmann-Voldseth and Dashiell, 2013). *Orius majusculus* is a polyphagous
69 predator with potential to control a considerable number of pest species, including whiteflies (Arnó
70 et al. 2008), aphids, and thrips (Butler and O'Neil, 2008). *Dolania coriaria* is a soil-dwelling

71 polyphagous predator that is an effective biological control agent of certain small soft-bodied
72 greenhouse pests (Carney et al., 2002). *Aphidoletes aphidimyza* has aphidophagous larvae and is
73 commonly used for biological control in greenhouses (van Schelt et al., 2000); the larvae go into the
74 soil to pupate or to hibernate (Harris, 1973).

75 Most studies use mortality as the only parameter to evaluate the effect of microbial control
76 agents against both target arthropod pests (e.g. Jandricic et al., 2014; Savitha et al., 2015; Eidy et
77 al., 2016) and non-target beneficial arthropods (e.g. Saito and Brownbridge, 2016); this is
78 particularly true when the relative effects of several of these agents being used together are
79 assessed to select the 'best' combination within an IPM context (Desneux et al., 2007). However,
80 understanding a variety of fitness-reducing (i.e. sublethal and/ or premortality) non-target effects
81 of microbial control agents on beneficial arthropods is indispensable in order to optimize IPM
82 programs that include the use of multiple natural enemies.

83 We hypothesized that differences in the biology and life cycles of the four chosen predatory
84 arthropods would lead to different levels of contact with soil, and thus different levels of exposure
85 and degrees of reduced fitness when that soil is inoculated with a microbial control agent. The
86 present laboratory study was established as part of the EU FP7 project INBIOSOIL and aimed to
87 assess the non-target effects of *M. brunneum* on four taxonomically different predatory arthropods,
88 when applied in soil, and measured by the fitness parameters: mortality, longevity, and fecundity.

89 **2. Materials and methods**

90 *2.1. Source and maintenance of insects*

91 Cohorts of all the arthropods were reared by EWH BioProduction and maintained at $23 \pm 0.5^\circ\text{C}$,
92 50-75% relative humidity, and L16: D8 light regime, complying with the IOBC quality control
93 guidelines for beneficial arthropods (van Lenteren, 2003). Newly emerged adults of *G. aculeifer*, *O.*
94 *majuscules*, and *D. coriaria*, or last instar larvae of *A. aphidimyza* were used in the experiments.
95 Cohorts were fed on *Tyrophagus putrescentiae* (Shrank) (Astigmata: Acaridae), *Ephestia kuhniella*
96 Zeller (Lepidoptera; Pyralidae) eggs, shell-free shrimp food (Ocean Nutrition, Newark, CA, United
97 States) and *Megoura viciae* (Buckt.) (Hemiptera; Aphididae), respectively. The experimental work
98 was done at the University of Copenhagen, Department of Plant and Environmental Sciences
99 (UCPH), and cohorts were maintained under the same conditions as used by EWH BioProduction.

100 The use of controlled cohorts ensured that all individuals evaluated were the same age and reared
101 under the same conditions.

102

103 2.2. *Source and preparation of the microbial inoculum*

104 *Metarhizium brunneum* strain KVL 12 – 19, which is the same genotype as GranMet/BIPESCO 5,
105 is held in long-term cryo-storage (-80°C) at the University of Copenhagen, Department of Plant and
106 Environmental Sciences. Stock cultures were grown on 4% Sabouraud dextrose agar (SDA; Merck,
107 Sweden) in Petri dishes and then stored at 8°C for up to six months prior to use. Subcultures for
108 experimental use were grown by transferring conidia from a stock culture plate onto SDA plates and
109 incubating at 20 ± 1°C for 20 days. Conidia were harvested by flooding the cultures with sterile 0.05%
110 Triton-X 100 (VWR, Sweden), and scraping with a sterile Drigalski spatula. The resulting suspension
111 was transferred to 50 ml stock tubes, and the conidial concentration of the stock suspension
112 determined using a hemocytometer (Fuchs-Rosenthal 0.0625 mm², depth 0.200 mm, VWR,
113 Sweden). Germination tests were made and conidia were only used when viability was > 95%. Stock
114 suspensions of conidia were refrigerated and used one day after preparation.

115

116 2.3. *Dipping trial*

117 Groups of twenty individuals (mixed sexes) from each species, except *A. aphidimyza*, were each
118 dipped into 1x10⁷ *M. brunneum* conidial suspensions (15-20ml) for 30 seconds; the suspension was
119 removed by vacuum filtration in a filter paper-lined Büchner funnel (Goettel and Inglis, 1997). The
120 inoculated predatory arthropods were then incubated individually at 22-23°C in a 16:8 light: dark
121 regime; to determine longevity, survival was recorded daily during a specific time determined by
122 results from pilot studies. The same number of individuals of each species were dipped in water
123 containing 0.05% Triton X-100 as the control; there was one replicate treatment group and one
124 replicate control group for each species and the experiment was repeated on three separate
125 occasions. Since the aim of this trial is to compare longevity following conventional inoculation, its
126 results are presented together with soil inoculation results.

127

128 2.4. *Exposure to conidia in agricultural soil*

129 2.4.1 Experimental set-up

130 Newly emerged adults (mixed sexes) of *G. aculeifer* (n = 20 per replicate container), *O.*
131 *majusculus* (n = 10 per replicate container), or last instar larvae of *A. aphidimyza* (n = 20 per replicate
132 container) were exposed to *M. brunneum* in soil; pilot experiments showed that *D. coriaria* has a
133 pre-oviposition period of 8 days, therefore, individual adults (n = 10 per replicate container) were
134 matured for this period before soil exposure. On each occasion that the experiment was run a
135 different species was evaluated and there were three replicate treatment containers and three
136 replicate control containers; the experiment was run on 3-5 separate occasions for each species to
137 increase replication and on each occasion mortality, cause of mortality (fungal outgrowth),
138 longevity, and fecundity were recorded for each individual.

139 Soil was obtained from the university experimental farm Bakkegaarden, which has been
140 managed as an organic farm for at least ten years. Each time the experiment was run, soil was sieved
141 through a 3mm mesh and 200g placed into a 10-15 L plastic bag. 10ml of conidial suspension ($1 \times$
142 10^8 conidia/ml) (to achieve a final concentration of 5×10^6 conidia/g of soil) was added to the soil
143 surface, and the bag was closed and mixed thoroughly. The same thing was done to provide control
144 soil except that inoculum was replaced with 0.05% Triton X-100. Treatment and control soils were
145 maintained at room temperature overnight and, before use, sieved again through a 3mm mesh to
146 ensure an even conidial distribution in the treatment soil. Inoculated soil (65g) was placed into each
147 of three replicate containers (155mL transparent cups with perforated lids, 6cm deep and 5cm
148 diameter at the widest part); the base of each container was previously covered with 5 mL water
149 agar (1.5%) to ensure a stable relative humidity during experiments (95% – 97% RH). Three control
150 containers were established in the same way using the uninoculated soil.

151 *Gaeolaelaps aculeifer*, *O. majusculus* and *D. coriaria* were exposed to soil in the lidded
152 containers and incubated at 23 ± 0.5 °C in a 16: 8 h light: dark regime for 3 days. The containers
153 were turned upside down once daily to ensure movement of the predators through the soil. Since
154 *O. majusculus* spent the majority of their time at the top of the container, beneath the perforated
155 lid where ventilation holes would likely reduce humidity, replicates of this species were inverted for
156 the first 24 h, to ensure that individuals remained near the water agar (higher humidity) during
157 possible fungal infection. After soil exposure, the predators were transferred individually into new
158 containers (30 ml) containing food; the base of each container was covered with 3ml of 1.5% water
159 agar to maintain a constant humidity. These containers were also sealed with a perforated lid to

160 allow ventilation, and all containers were incubated at the same temperature and light conditions
161 as before. Predators were transferred to new containers with fresh diet every 2nd or 3rd day to avoid
162 growth of saprophytic fungi on the diet. *Geolaelaps aculeifer* was fed on *Ephestia kuhniella* eggs,
163 which are known to be a good-quality prey for this species. *Orius majusculus* was also fed on *E.*
164 *kuhniella* eggs, as in the cohort rearing. *Dalotia coriaria* was fed on shell-free shrimp fish food, as
165 was used in the cohort rearing. Last instar *A. aphidimyza* larvae (5th instar) were exposed to soil in
166 the same type of perforated containers as the other predatory species and incubated under the
167 same conditions. However, following introduction they began to burrow into the soil immediately
168 for pupation and remained there until the first emerging adults could be observed (usually day 12).
169 The emergence period was not more than 3 days, and during this period the number of emerged
170 females and males was recorded daily.

171

172 2.4.2 Mortality, longevity and *Metarhizium* outgrowth

173 The experimental set up described in 2.4.1 was used. All individuals of all species, except *A.*
174 *aphidimyza*, were checked daily or every second day, depending on the species. Dead predators,
175 from both treated and control groups, were transferred to unventilated containers (30 ml) with
176 1.5% water agar, and incubated to allow mycosis to develop (fungal sporulation from a cadaver).
177 Three factors were recorded: a) mortality: the day of death of an individual, b) longevity: how long
178 each individual survived after soil exposure until the end of the experiment and c) mycosis amongst
179 dead individuals clearly identified as an outgrowth of *Metarhizium*. *Metarhizium* outgrowth was not
180 recorded adult female *A. aphidimyza* since a pilot study had shown that emerging females were
181 never infected. The experiment was repeated on three occasions.

182

183 2.4.3 Fecundity of beneficial predators

184 The experimental set up described in 2.4.1 was used. The number of days necessary for each
185 species to mate was established after pilot studies.

186

187 2.4.3.1 Fecundity of *Geolaelaps aculeifer*

188 After the initial 3 days of soil exposure, each female mite was paired with a male mite from
189 the same replicate and allowed to mate for 48 h; the female was then moved to a new container

190 (30 ml) with 1.5% water agar to record fecundity. Females were transferred to new containers with
191 food and oviposition sites and the number of eggs laid was recorded every 48 hours for 10 days.
192 After 24 days, the experiment was terminated. The experiment was repeated on three occasions.

193 2.4.3.2 Fecundity of *Orius majusculus*

194 After the initial 3 days of soil exposure, each female was paired with a male from the same
195 replicate and allowed to mate in an empty container (30 ml); mating normally happened within a
196 few minutes (15-30 min). Females were then placed individually in ventilated containers (30 ml)
197 with 1.5% water agar, provided with *E. kuhniella* eggs as food and a 2cm piece of a green bean as
198 an oviposition site. Organic beans were used that had been washed in soapy water (perfume-free).
199 Females were transferred to new containers and the number of eggs laid was recorded every 48
200 hours for 12 days. After 24 days, the experiment was terminated. The experiment was repeated on
201 four occasions.

202 2.4.3.3 Fecundity of *Dalotia coriaria*

203 After the 3 days of soil exposure, adults were briefly anesthetized with CO₂ to be sexed, as
204 this requires a visual inspection of the 8th abdominal sternite under a stereomicroscope. Each female
205 was paired with a male from the same replicate in ventilated containers (30 ml) with 1.5% water
206 agar and, in addition to the diet, a small amount of dried sphagnum was provided to protect
207 offspring from cannibalism. Every 48 hours for 25 days, adults were moved to a new container and
208 the old container was incubated at 23 °C to allow larvae to hatch because eggs were too difficult to
209 see. After 6 days first instar larvae could be observed and the number recorded. The experiment
210 was terminated after 25 days at which time the adults were again sexed to ensure that the initial
211 identification had been correct in cases where eggs were not found. The experiment was repeated
212 on five occasions.

213

214 2.4.3.4 Fecundity of *Aphidoletes aphidimyza*

215 Pairs of females and males that were from the same container and had emerged on the same
216 day, were transferred to new containers with 1.5% water agar and a piece of filter paper dipped in
217 a 1:10 water: organic honey solution. If there were more than ten emerging adults, the number was
218 evenly distributed over two cups, always ensuring that there were males and females in each

219 container. After 24 hours, females were transferred individually to new containers (155 ml) with 1.5
220 ml agar and a barley leaf infested with 5-10 adult *Rhopalosiphum padi* (L.) aphids. Four days after
221 female emergence, the number of *A. aphidimyza* eggs was recorded. This experiment was repeated
222 on four occasions, with a total of 320 *A. aphidimyza* larvae.

223

224 2.5 Data analysis

225 Data were analyzed in the statistical software package SAS (Version 9.4, SAS Institute, 2015).
226 For *G. aculeifer*, *O. majusculus* and *D. coriaria* the effect of treatment on mortality in both the
227 dipping trial and the soil exposure experiment, was analyzed by a chi-square test and in a
228 generalized linear mixed model (GLMM) (proc GLIMMIX) assuming a binomial distribution with a
229 random effect of experimental repetition (block effect). The odds ratios obtained from logistic
230 regression analysis were used to estimate the relative risk of mortality. The effect of treatment on
231 longevity was analyzed using the nonparametric proc LIFETEST which computes estimates of the
232 survival distribution function. We used the life-table method of computing estimates. Proc LIFETEST
233 provides two statistical analyses, the modified Wilcoxon test which is particularly sensitive to
234 differences in the early part of the curves and log-rank test which is more sensitive to the later part
235 . Significance ($p < 0.05$) in one test was regarded as sufficient to accept that there was a true
236 significant difference. Proc LIFETEST allows for right-censored data, and was used for the few
237 individuals accidentally lost during the experiment and for individuals still alive when the
238 experiment was terminated. The effect of treatment on *Metarhizium* outgrowth on dead insects
239 was compared amongst the four species and tested using a chi-square test ($p < 0.05$). For each
240 species, the total number of eggs laid (fecundity) as an effect of treatment was analyzed using a
241 generalized linear mixed model (proc GLIMMIX) assuming a negative binomial distribution with a
242 random effect of experimental repetition (block effect). The fixed effects were tested in a 2-way
243 design between species and treatment, and comparisons between treatments were made using
244 least squares means. Likewise mean daily number of eggs was analyzed using a a generalized linear
245 mixed model (proc GLIMMIX) assuming a negative binomial distribution with a random effect of
246 experimental repetition (block effect), using the same fixed effects as for total number of eggs laid.
247 For *A. aphidimyza*, the adult life span is very short, and the experimental design had to be adjusted
248 because the life stage exposed to soil was the pupal stage. Midge emergence was used to assess

249 pupal mortality. Both mortality and fecundity were analyzed using a GLMM (proc GLIMMIX)
250 assuming a binomial distribution with a random effect of experimental repetition (block effect).
251 Additionally, a random effect of the set-up was included to account for overdispersion of the data.

252

253 **3 Results**

254 3.1 *Effects of either dipping or exposure to M. brunneum in the soil on fitness attributes of*
255 *Geolaelaps aculeifer, Orius majusculus and Dalotia coriaria*

256 3.1.1 Mortality following exposure to *M. brunneum* in the soil

257 Neither the chi-square test (Table 1) nor the GLIMMIX analysis showed a significant lethal
258 effect of soil exposure to fungus on *G. aculeifer* ($F_{1, 2} = 0.01$, $P = 0.913$). *Orius majusculus* mortality
259 was significantly higher after exposure to fungus in the soil than in the control group ($F_{1, 3} = 28$, $P =$
260 0.013) and the relative risk of death for *O. majusculus* was 103 times higher in the treatment than
261 in the control (Table 1). According to the GLIMMIX analysis, *Dalotia coriaria* mortality was not
262 significantly affected by exposure to fungus in the soil ($F_{1, 4} = 5.36$, $P = 0.081$), but the chi-square test
263 did show a significant effect (Table 1) with the relative risk of death being 3.8 times higher in the
264 treatment than in the control (Table 1).

265

266 3.1.2 *Metarhizium* outgrowth on cadavers following exposure to *M. brunneum* in the soil

267 *Geolaelaps aculeifer*, *O. majusculus*, and *D. coriaria* treated with *Metarhizium* showed fungal
268 outgrowth in 20%, 83.3% and 57.2% of the cadavers ($\chi^2 = 37.52$, 2 df, $p < 0.0001$), respectively.
269 *Geolaelaps aculeifer* had significantly fewer cadavers that produced fungal outgrowth than the
270 other two species ($\chi^2 = 19.86$, 2 df, $p < 0.0001$). No fungal outgrowth was observed amongst the dead
271 individuals from the control groups.

272

273 3.1.3 Longevity following either dipping or exposure to *M. brunneum* in the soil

274 Dipping did not affect longevity (as measured by survival) of *G. aculeifer* (treated $n = 48$;
275 control $n = 48$) (log-rank: $\chi^2 = 0.12$, 1 df, $p = 0.722$; Wilcoxon: $\chi^2 = 0.35$, 1 df, $p = 0.551$) (Fig. 1A) and *D.*
276 *coriaria* (treated $n = 96$; control $n = 96$) (log-rank: $\chi^2 = 1.36$, 1 df, $p = 0.243$; Wilcoxon: $\chi^2 = 1.34$, 1 df,
277 $p = 0.246$) compared with the control (Fig. 1E). However, *O. majusculus* longevity (treated $n = 98$;
278 control $n = 96$) was significantly reduced after dipping compared with the control (log-rank: $\chi^2 = 4.83$,

279 1 df, $p=0.027$; Wilcoxon: $\chi^2= 4.71$, 1 df, $p= 0.03$) (Fig 1C). Longevity was not significantly reduced
280 after fungal exposure in soil for *G. aculeifer* (Fig. 1B) (log-rank: $\chi^2= 2.93$, 1 df, $p=0.0867$; Wilcoxon:
281 $\chi^2= 1.31$, 1 df, 0.252) compared with the control. There was a highly significant effect of exposure
282 to fungus in the soil on *O. majusculus* (log-rank: $\chi^2= 28.56$, 1 df, $p< 0.0001$; Wilcoxon: $\chi^2= 28.62$, 1
283 df, $p< 0.0001$; Fig 1D) and a significant effect of exposure to fungus in the soil on *D. coriaria* (log-
284 rank: $\chi^2= 4.80$, 1 df, $p=0.028$; Wilcoxon: $\chi^2= 4.89$, 1 df, $p= 0.027$; Fig 1F) compared with the controls.

285

286 3.1.4 Fecundity following exposure to *M. brunneum* in the soil

287 Exposure to fungus in the soil significantly reduced the fecundity (total number of laid eggs) of
288 *O. majusculus* and *D. coriaria* ($F_{1, 416}= 13.85$, $P= 0.0002$ and $F_{1, 416}= 6.27$, $P= 0.013$, respectively),
289 decreasing the number of offspring. However, exposure to fungus in the soil did not significantly
290 reduce *G. aculeifer* fecundity ($F_{1, 416}= 0.00$, $P= 0.957$; Fig. 2) compared with the control. Mean daily
291 fecundity was also reduced significantly by treatment for *D. coriaria* (mean \pm SE for control: $1.09 \pm$
292 0.18 and treated: 0.64 ± 0.08 , $F_{1, 416}= 7.23$, $P=0.0075$), but for *O. majusculus* daily fecundity was only
293 marginally and not significantly reduced by treatment (mean \pm SE for control: 10.75 ± 0.97 and
294 treated: 8.99 ± 0.83 ; $F_{1, 416}= 13.85$, $P= 0.054$), while exposure to fungus in the soil did not significantly
295 reduce *G. aculeifer* mean daily fecundity compared with the control (mean \pm SE for control: $4.21 \pm$
296 0.14 and treated: 4.18 ± 0.14 ; $F_{1, 416}= 0.01$, $P= 0.93$).

297

298 3.2 Effects of exposure to *M. brunneum* in the soil on fitness attributes of *Aphidoletes aphidimyza*

299 Dead larvae/ pupae could not be recovered from the soil, so larval mortality was calculated as
300 the difference between the number of adults emerging and the number of larvae that had been
301 introduced into the soil. Larval mortality levels were significantly higher when exposed to fungus in
302 the soil compared with the control ($F_{1, 23}= 33.99$, $P< 0.0001$) with 59.4% of the larvae being dead
303 compared with 40.7% dead in the control ($\chi^2= 12.22$, 1 df, $p< 0.0005$). However, amongst emerged
304 females, the number of eggs/female was not significantly affected by exposure to fungus in the soil
305 compared with the control ($F_{1, 78.76}= 0.50$, $P= 0.480$; Fig. 2).

306

307 4 Discussion

308 In the present study, effects of *M. brunneum* strain BIPESCO 5 on mortality, longevity, and
309 fecundity of four predatory arthropods - *G. aculeifer*, *O. majusculus*, *D. coriaria* and *A. aphidimyza* -
310 were examined under laboratory conditions. The bioassays were designed to simulate the exposure
311 of each predator to high doses of the entomopathogenic fungus in their natural environment, the
312 soil, thus evaluating a worst-case scenario. All three fitness parameters were assessed on the same
313 individuals.

314 Even when it is not fatal, a fungal infection may have sublethal, non-target effects on the
315 performance of natural enemies. Non-target effects may be expressed as changes in; the lifespan
316 of beneficial arthropods (through altered developmental rates); population growth (through
317 reduced fecundity); or behavior (Ormond et al., 2011; Wu et al., 2015; Jarrahi and Safavi, 2016). A
318 meta-analysis study showed that predator longevity, fecundity, and survival decreased by 26%, 31%,
319 and 13% respectively, when predators consumed pathogen-infected prey, demonstrating that
320 infected prey were a low-quality resource (Flick et al., 2016).

321 In this study, the species that was least affected by fungal exposure in the soil was the soil-living
322 *G. aculeifer*; neither mortality, longevity nor fecundity were affected by fungal exposure. Even
323 though many studies have shown the efficacy of this soil-living predatory mite species against
324 important insect pests, this is the first study assessing the interaction between *G. aculeifer* and
325 entomopathogenic fungi. For another species, of the same genus, *G. gillesspiei*, when exposed to *M.*
326 *brunneum* on filter paper, mortality was 28% higher than in the control (Saito and Brownbridge,
327 2016). High tolerance in mites to entomopathogenic fungi was also found in another study in which
328 two mite species, *Amblyseius swirskii* Athias-Henriot and *Neoseiulus cucumeris* (Oudemans), were
329 used in combination with the entomopathogenic fungus *Beauveria bassiana* (Bals.-Criv) Vuill.
330 against the pest *Diaphorina citri* Kuwayama (Zhang et al., 2015).

331 The species most negatively affected by fungal exposure in the soil was *O. majusculus* with the
332 highest mortality rate and most reduced fecundity compared with the control. However, mean daily
333 fecundity was only marginally and not significantly reduced, indicating that reduced total fecundity
334 was principally an effect of shorter life, when infected.

335 Only few studies exist regarding the effects of entomopathogenic fungi on anthocorid predators.
336 One of them shows that the presence of both generalist and specialist entomopathogenic fungi
337 differently affects the prey handling time of *O. majusculus* as well as its predation rate (Jacobsen

338 S.K. personal communication.). The species *O. albidipennis* responded to the presence of
339 *Metarhizium anisopliae* (Metchn.) Sorokin on hosts by increasing searching time and decreasing
340 feeding time and predation rate (Pourian et al., 2011). Furthermore, when *B. bassiana* was applied
341 directly to *Orius sauteri* (Poppius) there was no increase its mortality or longevity, but when *O.*
342 *sauteri* was fed on *B. bassiana*-infected *Frankliniella occidentalis* Pergande larvae its longevity was
343 approximately 10-15% shorter than the control, although this was not statistically significant (Gao
344 et al., 2012). However, since *Orius majusculus* does not normally come into contact with soil during
345 its life cycle, a semi-field or pot trial would be needed to assess more realistically the side-effects.

346 *Dalotia coriaria* had only a slightly, though statistically significant, reduction in fecundity and
347 increase in mortality when exposed to *M. brunneum*. However, its survival rate was still as high as
348 92.71% in the treated group, in this laboratory experiment, which did represent a worst-case
349 scenario. This indicates that a low to negligible side effect of *M. brunneum* can be expected in a field
350 situation over the time span studied. The experiment was terminated when the beetles were about
351 30 days old, and the effect of treatment was observed, but it is possible that mortality would have
352 increased more in the treated individuals as *D. coriaria* adult longevity is around 60 and 48 days for
353 males and females, respectively (Echegaray and Cloyd, 2013). Another study showed that *M.*
354 *brunneum* strain F52, applied in a growing medium, was not harmful to *D. coriaria* because mortality
355 and feeding capacity were not affected by the treatment (Cloyd et al., 2009), while a recent study
356 using the same strain of *M. brunneum* inoculated on a filter paper found that the mortality of *D.*
357 *coriaria* was 35% higher in the fungal treated group than in the control (Saito and Brownbridge,
358 2016).

359 As a result of higher larval mortality after exposure to *M. brunneum*, significantly fewer *A.*
360 *aphidimyza* midges emerged from the fungal treated soil than from the control soil. Amongst those
361 females that emerged, fecundity was not affected by treatment. Our previous greenhouse study
362 showed that the number of *A. aphidimyza* midges emerging from *M. brunneum*-treated soil and the
363 number of eggs laid were not affected by fungal presence; however, the number of midges was four
364 times higher in the control than in the treatment at the end of the experiment (Azevedo et al., 2017).

365 The effect of microbial biological control agents on beneficial arthropods has been the focus of
366 a number of studies. However, our study is innovative because we assessed the non-target effects
367 of an entomopathogenic fungus, applied in soil, on different classes of arthropods and orders of

368 insects, consequently covering differences in the effect of fungal exposure on different parts of the
369 species' life cycles. A further relevant aspect of the study was to investigate the non-target effects
370 in the soil, and not only the effects of direct application. The entomopathogenic fungal dose used
371 was higher than that used in field conditions and was applied under optimal controlled conditions;
372 therefore, the four species of predator were evaluated under worst-case scenario conditions.

373 According to the working group 'Pesticides and Beneficial Organisms' of the International
374 Organization for Biological Control (IOBC), Western Palearctic Regional Section (IOBC-WPRS), an
375 insecticide can be described as harmless (< 30% mortality), slightly harmful (30–79% mortality),
376 moderately harmful (80–99% mortality) and harmful (>99% mortality) when evaluated under
377 laboratory conditions by direct application (Sterk et al., 1999). Considering these generally accepted
378 thresholds, we conclude that *M. brunneum* isolate BIPESCO 5, when applied to the soil, is harmless
379 to *G. aculeifer* and moderately harmful to *O. majusculus*. As it is unlikely that *O. majusculus* will have
380 significant contact with the soil during its life cycle, we expect that, in a field situation, *O. majusculus*
381 will be at low risk of infection by *M. brunneum* in the soil. *Dalotia coriaria* and *A. aphidimyza* have
382 an intermediate response to *M. brunneum* isolate BIPESCO 5, which could be considered as harmless
383 and slightly harmful to these two species, respectively. Both species naturally have sporadic contact
384 with the soil, so they may be better adapted to tolerate exposure to microorganisms.

385 The four species of predator selected represent a range of natural enemy taxa with different
386 levels of soil contact and so also provide a practical model for testing potential non-target effects
387 on natural enemies.

388

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398 experimental design; executed part of the experiments. Jørgen Eilenberg: Contributed with ideas
399 for the setup and experimental design; revised the manuscript. Lene Sigsgaard: Analyzed the data;
400 contributed with ideas for the setup and experimental design; revised the manuscript.

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538

539 **Legends**

540

541 **Figure 1.** Plots of survival probability estimated for *G. aculeifer*, *O. majusculus* and *D. coriaria* after dipping
542 test in a *Metarhizium* suspension with 1×10^7 conidia per milliliter ○ and control ● (A) and exposure to $5 \times$
543 10^6 conidia of *M. brunneum* per gram of soil ○ and control ● (B).

544

545 **Figure 2.** Mean number of eggs (+ SE) laid by *G. aculeifer*, *O. majusculus*, *D. coriaria* and *A. aphidimyza* in
546 the control ($n= 104$; $n= 50$; $n= 62$ and $n= 84$, respectively) and following exposure to *M. brunneum* in the
547 soil ($n= 93$; $n= 55$; $n= 58$ and $n= 84$, respectively). Columns with the sign (*) are significantly different
548 (binomial GLMM, $P < 0.05$).

549

550 **Table 1.** *Geolaelaps aculeifer*, *Orius majusculus* and *Dalotia coriaria* mortality, which is the proportion of
551 dead individuals during the experiment, after exposure to soil inoculated with *Metarhizium brunneum*. Data
552 were pooled from three replicate experiments and analyzed using a chi-square test ($\alpha = 0.05$).

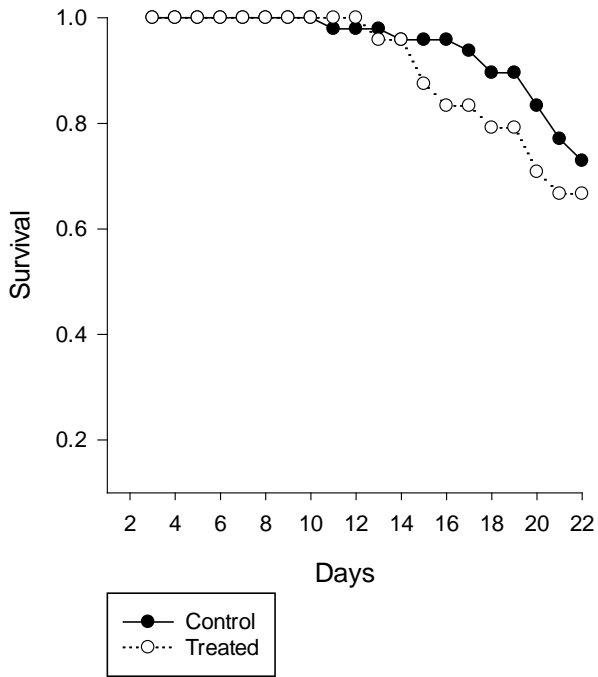
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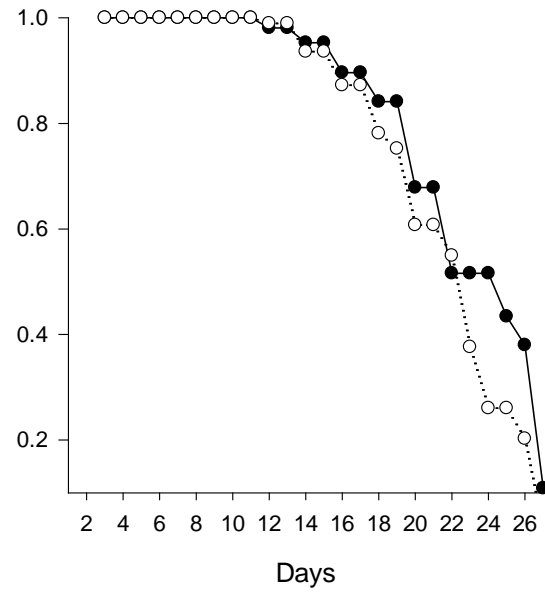
Table 1: *Geolaelaps aculeifer*, *Orius majusculus* and *Dalotia coriaria* mortality, which is the proportion of dead individuals during the experiment, after exposure to soil inoculated with *Metarhizium brunneum*. Data were pooled from three replicate experiments and analyzed using a chi-square test ($\alpha = 0.05$).

Species	Treatment	n	Mortality	χ^2 test	Odds ratio
<i>G. aculeifer</i>	Control	106	43.4%	$\chi^2 = 0.4, 1 \text{ df}, p = 0.5258$	1.19
	Fungus	94	47.8%		
<i>O. majusculus</i>	Control	69	18.8%	$\chi^2 = 69, 1 \text{ df}, p < 0.0001$	103.38
	Fungus	50	96%		
<i>D. coriaria</i>	Control	197	2.3%	$\chi^2 = 6.09, 1 \text{ df}, p = 0.0135$	3.79
	Fungus	192	7.3%		

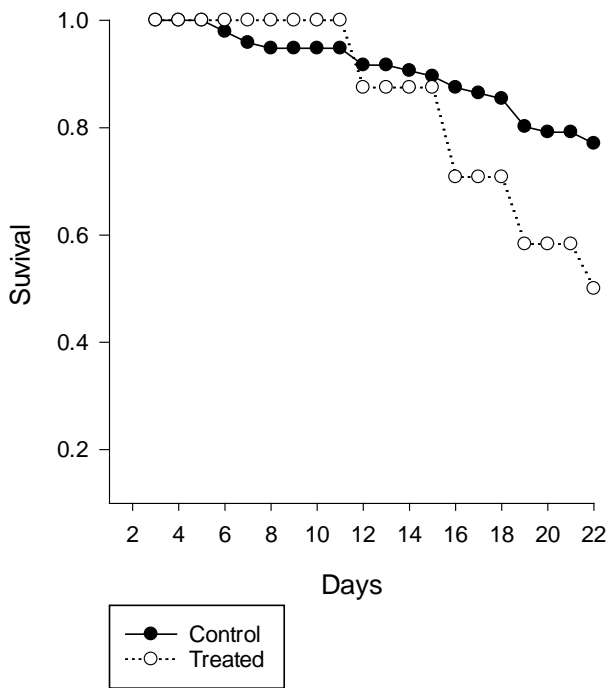
G. aculeifer (A)



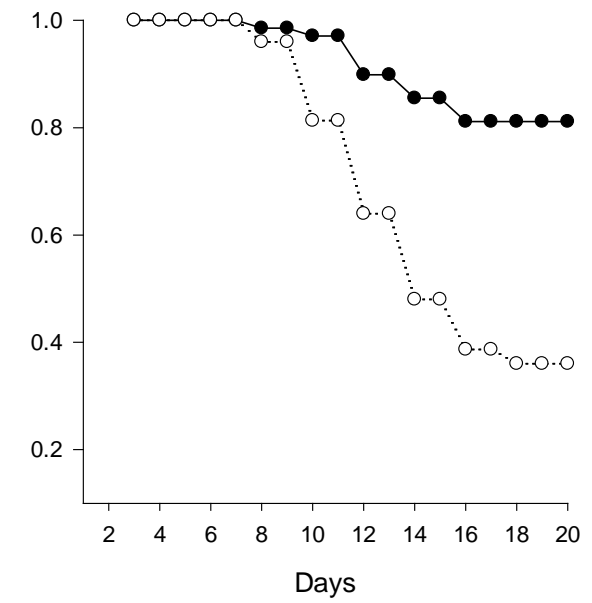
G. aculeifer (B)



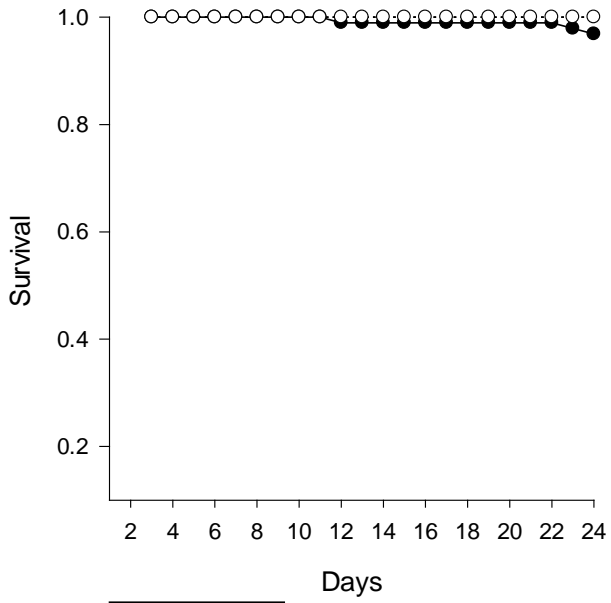
O. majusculus (C)



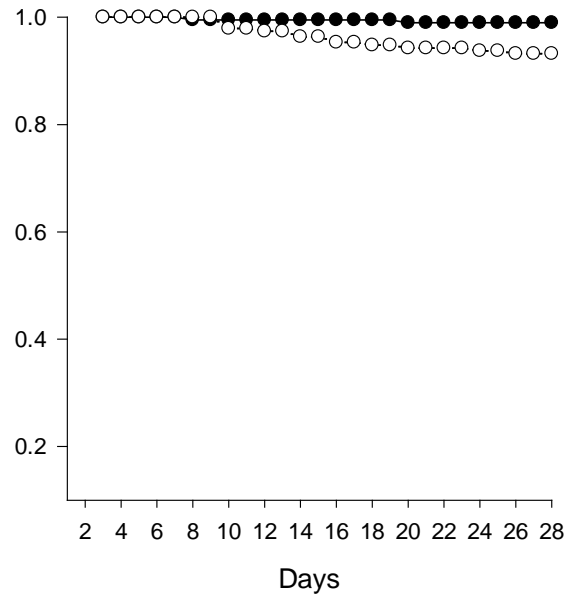
O. majusculus (D)

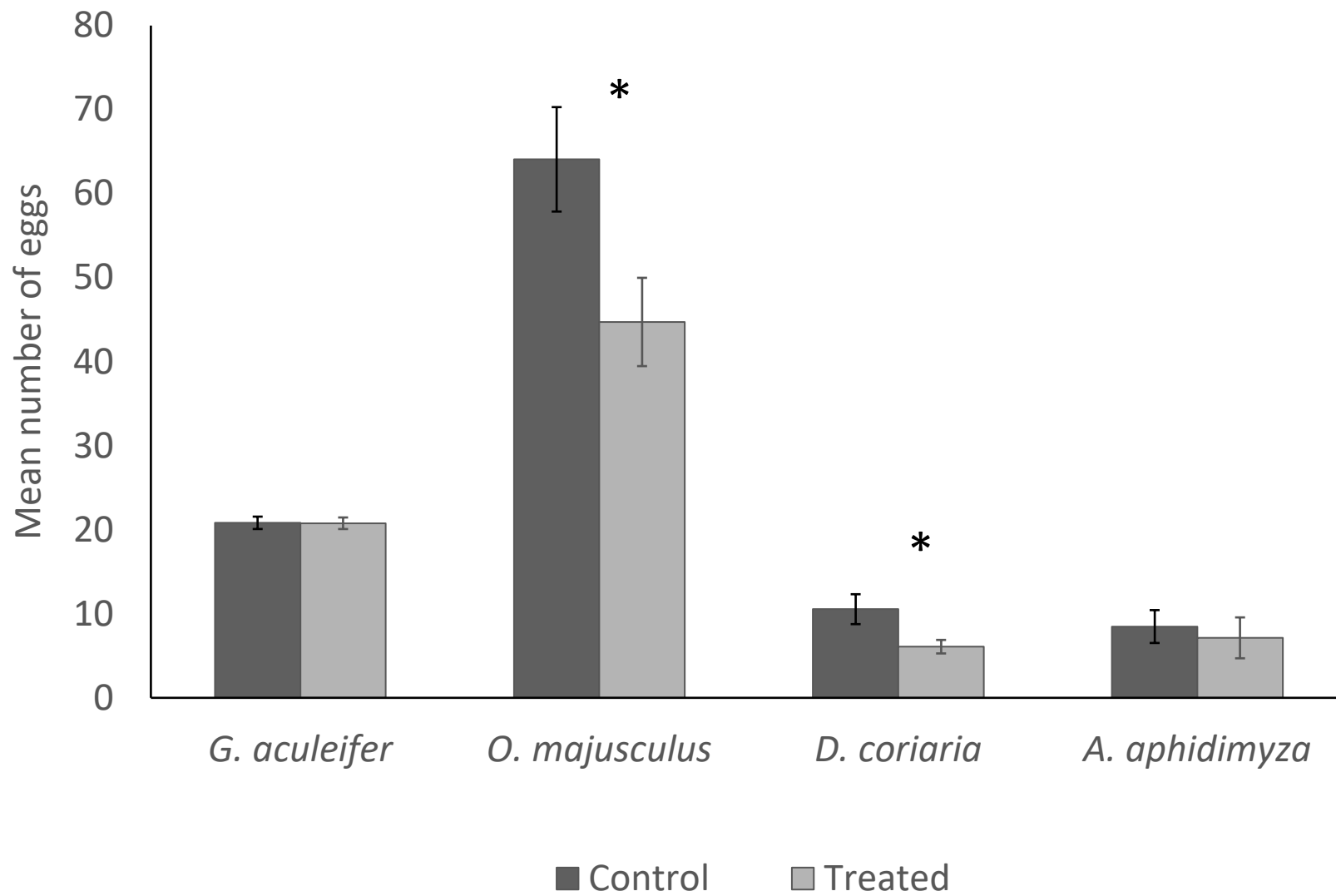


D. coriaria (E)



D. coriaria (F)





Aphidoletes aphidimyza
(Diptera, Cecidomyiidae)



Orius majusculus
(Hemiptera, Anthocoridae)

Atheta coriaria (Coleoptera, Staphylinidae)

Aphidoletes aphidimyza

Geolelaps aculifer (Acari,
Mesostigmata, Laelapidae)

Metarhizium brunneum
(Ascomycota: Hypocreales)

