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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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ABSTRACT

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

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. *Source and maintenance of insects*

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

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

2.2. *Source and preparation of the microbial inoculum*

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

2.3. *Dipping trial*

Groups of twenty individuals (mixed sexes) from each species, except *A. aphidimyza*, were each dipped into $1x10^7$ *M. brunneum* conidial suspensions (15-20ml) for 30 seconds; the suspension was removed by vacuum filtration in a filter paper-lined Büchner funnel (Goettel and Inglis, 1997). The inoculated predatory arthropods were then incubated individually at 22-23°C in a 16:8 light: dark regime; to determine longevity, survival was recorded daily during a specific time determined by results from pilot studies. The same number of individuals of each species were dipped in water containing 0.05% Triton X-100 as the control; there was one replicate treatment group and one replicate control group for each species and the experiment was repeated on three separate occasions. Since the aim of this trial is to compare longevity following conventional inoculation, its results are presented together with soil inoculation results.

2.4. *Exposure to conidia in agricultural soil*

2.4.1 Experimental set-up

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

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

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

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

2.4.2 Mortality, longevity and Metarhizium *outgrowth*

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

2.4.3 Fecundity of beneficial predators

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

2.4.3.1 Fecundity of *Geolaelaps aculeifer*

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

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

2.4.3.2 Fecundity of *Orius majusculus*

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

2.4.3.3 Fecundity of *Dalotia coriaria*

After the 3 days of soil exposure, adults were briefly anesthetized with CO_2 to be sexed, as this requires a visual inspection of the 8th abdominal sternite under a stereomicroscope. Each female was paired with a male from the same replicate in ventilated containers (30 ml) with 1.5% water agar and, in addition to the diet, a small amount of dried sphagnum was provided to protect offspring from cannibalism. Every 48 hours for 25 days, adults were moved to a new container and the old container was incubated at 23 ^{o}C to allow larvae to hatch because eggs were too difficult to see. After 6 days first instar larvae could be observed and the number recorded. The experiment was terminated after 25 days at which time the adults were again sexed to ensure that the initial identification had been correct in cases where eggs were not found. The experiment was repeated on five occasions.

2.4.3.4 Fecundity of *Aphidoletes aphidimyza*

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

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

2.5 Data analysis

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

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

3 Results

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

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

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

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

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

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

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

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

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

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

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

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

4 Discussion

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

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

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

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

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

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

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

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

The effect of microbial biological control agents on beneficial arthropods has been the focus of a number of studies. However, our study is innovative because we assessed the non-target effects 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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538

Legends

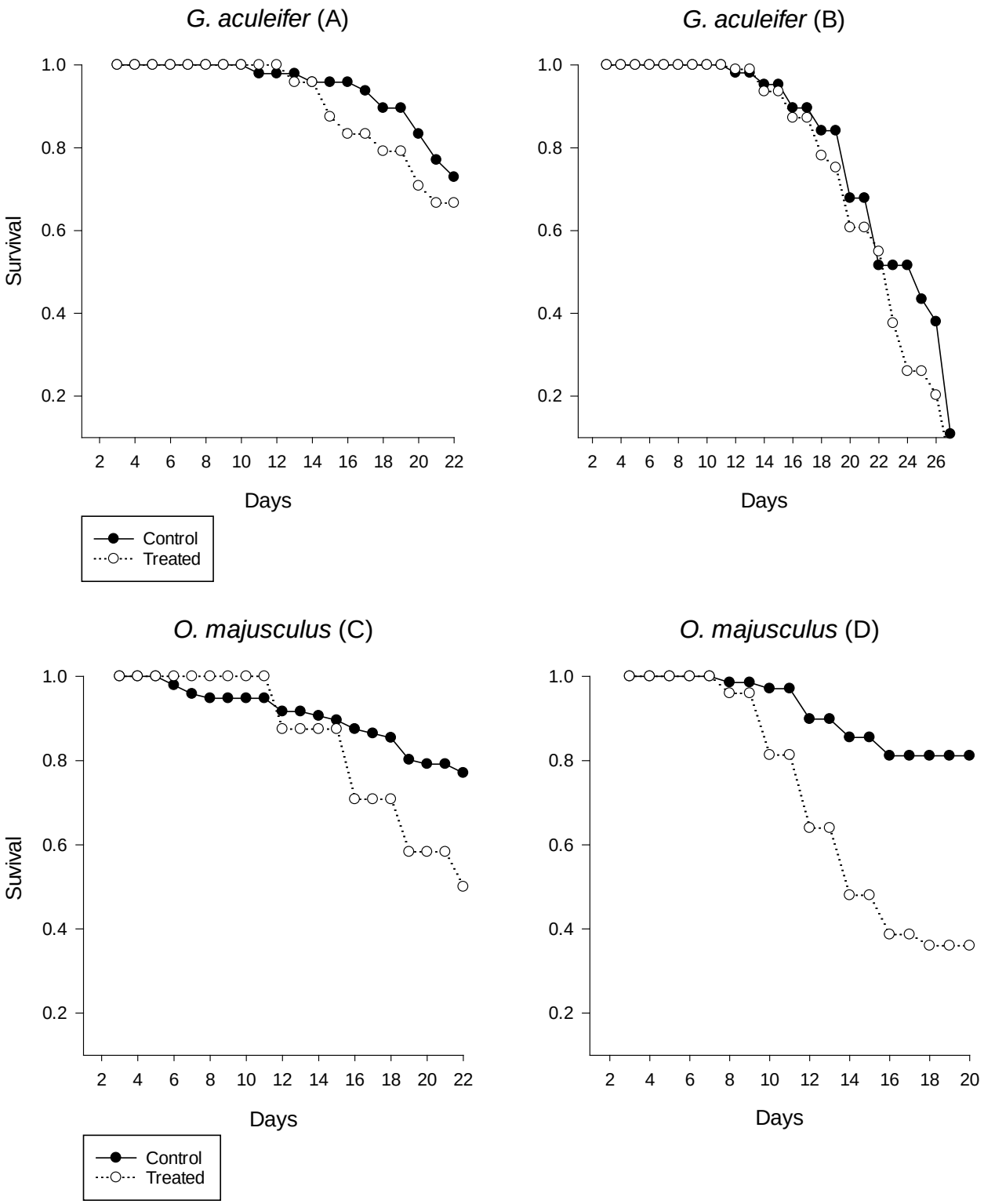
Figure 1. Plots of survival probability estimated for *G. aculeifer, O. majusculus* and *D. coriaria* after dipping test in a *Metarhizium* suspension with 1 x 10^7 conidia per milliliter ○ and control ● (A) and exposure to 5 x 10^6 conidia of *M. brunneum* per gram of soil ○ and control ● (B).

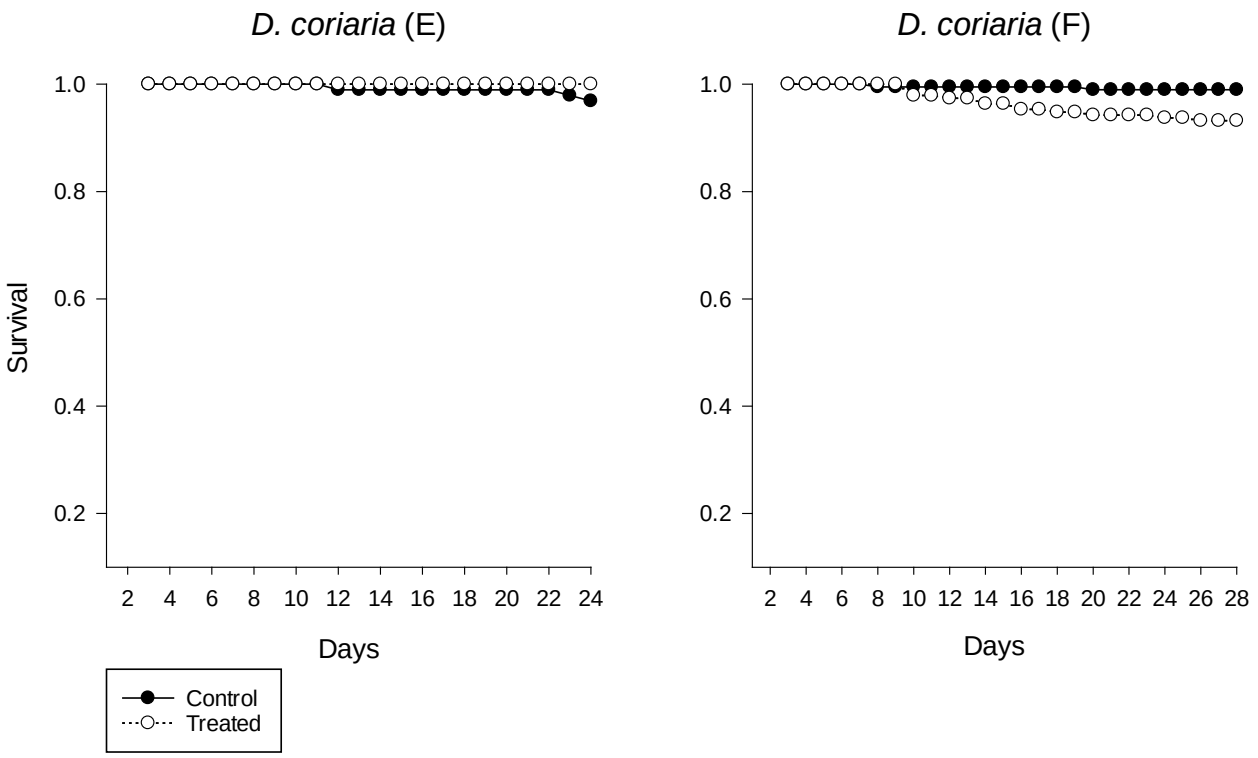
Figure 2. Mean number of eggs (+ SE) laid by *G. aculeifer, O. majusculus, D. coriaria* and *A. aphidimyza* in the control (*n*= 104; *n*= 50; *n*= 62 and *n*= 84, respectively) and following exposure to *M. brunneum* in the soil (*n*= 93; *n*= 55; *n*= 58 and *n*= 84, respectively). Columns with the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

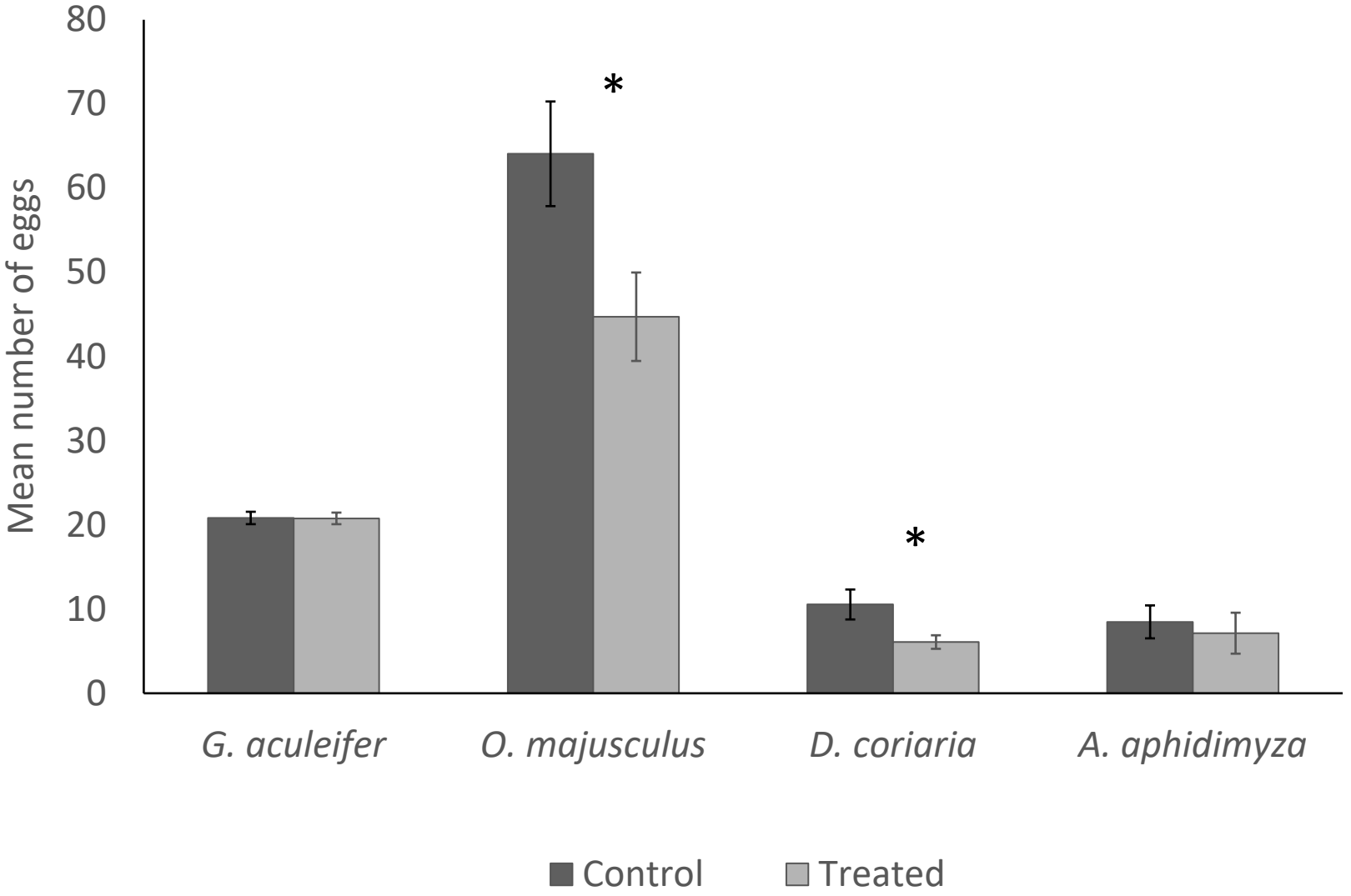
Table 1. *Geolaelaps aculeifer, Orius majusculus and Dalotia coriaria* mortality, which is the proportion of dead individuals during the experiment, after exposure to soil inoculated with *Metarhizium brunneum.* Data were pooled from three replicate experiments and analyzed using a chi-square test ($\alpha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

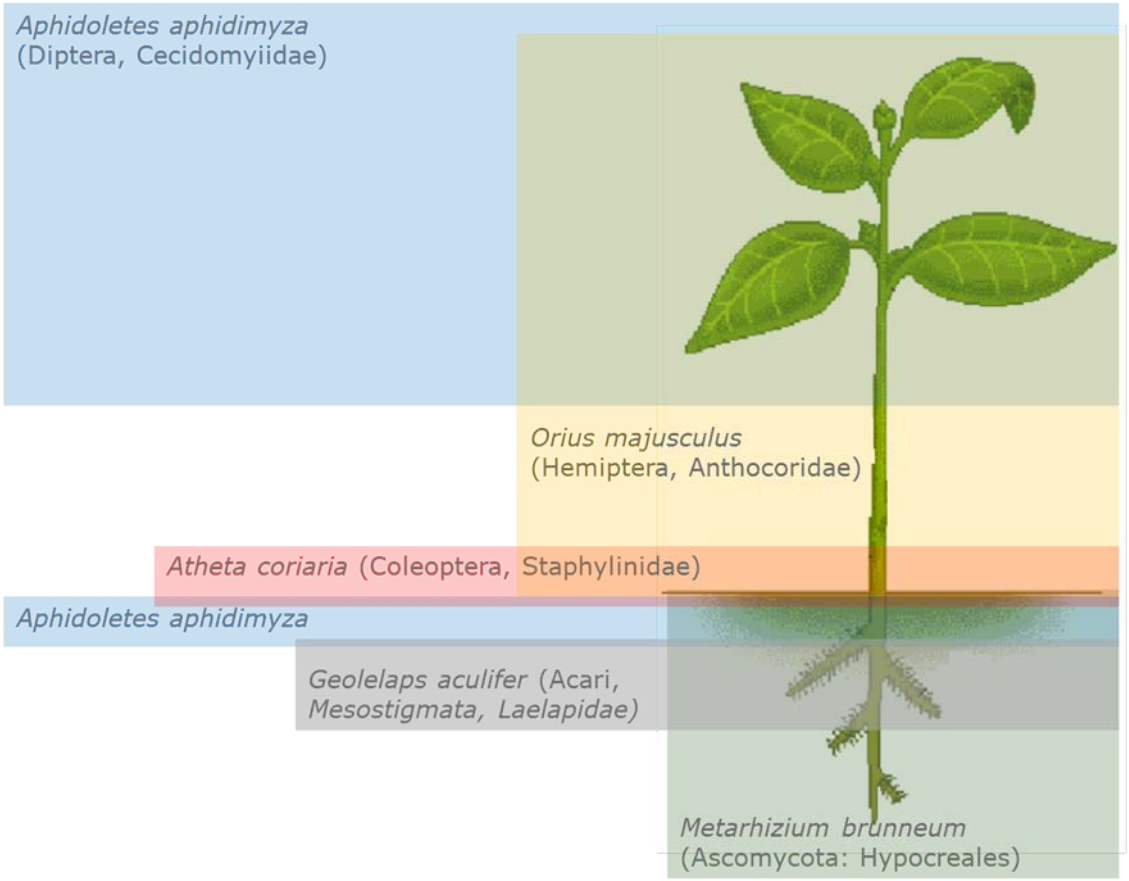
Table 1: *Geolaelaps aculeifer, Orius majusculus and Dalotia coriaria* mortality, which is the proportion of dead individuals during the experiment, after exposure to soil inoculated with *Metarhizium brunneum.* Data were pooled from three replicate experiments and analyzed using a chi-square test ($\alpha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

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


G. aculeifer (A)
G. aculeifer (B)
O. majusculus (C)
O. majusculus (D)
Survival
Suvival
Days
Control
Treated
1.0
0.8
0.6
0.4
0.2
2 4 6 8 10 12 14 16 18 20 22
2 4 6 8 10 12 14 16 18 20 22 24 26


D. coriaria (E)
D. coriaria (F)
Survival
1.0
0.8
0.6
0.4
0.2
2 4 6 8 10 12 14 16 18 20 22 24
2 4 6 8 10 12 14 16 18 20 22 24 26 28
Days
Days
Control
Treated


80
70
60
50
40
30
20
10
0
Mean number of eggs
*
*
G. aculeifer
O. majusculus
D. coriaria
A. aphidimyza
Control
Treated


Aphidoletes aphidimyza
(Diptera, Cecidomyiidae)
Orius majusculus
(Hemiptera, Anthocoridae)
Atheta coriaria (Coleoptera, Staphylinidae)
Aphidoletes aphidimyza
Geolelaps aculifer (Acari, *Mesostigmata, Laelapidae)*
Metarhizium brunneum
(Ascomycota: Hypocreales)