

1 **Mortality of native and invasive ladybirds co-infected by ectoparasitic and**  
2 **entomopathogenic fungi**

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19 **ABSTRACT:** *Harmonia axyridis* is an invasive alien ladybird in North America and Europe.

20 Studies show that multiple natural enemies are using *Ha. axyridis* as a new host. However, thus

21 far, no research has been undertaken to study the effects of simultaneous infections of multiple

22 natural enemies on *Ha. axyridis*. We hypothesized that high thallus densities of the ectoparasitic

23 fungus *Hesperomyces virescens* on a ladybird weaken the host's defenses, thereby making it

24 more susceptible to infection by other natural enemies. We examined mortality of the North  
25 American-native *Olla v-nigrum* and *Ha. axyridis* co-infected with *He. virescens* and an  
26 entomopathogenic fungus—either *Beauveria bassiana* or *Metarhizium anisopliae*. Laboratory  
27 assays revealed that *He. virescens*-infected *O. v-nigrum* individuals are more susceptible to  
28 entomopathogenic fungi, but *Ha. axyridis* does not suffer the same effects. This is in line with  
29 the enemy release hypothesis, which predicts that invasive alien species in new geographic areas  
30 experience reduced regulatory effects from natural enemies compared to native species.  
31 Considering our results, we can ask how *He. virescens* affects survival when confronted by other  
32 pathogens that previously had little impact on *Ha. axyridis*.

### 33 **Introduction**

34 In nature and in agricultural ecosystems, hosts may be exploited by more than one natural  
35 enemy. These organisms interact with each other and with their hosts<sup>1</sup>. These complex  
36 interactions shape the population structure and dynamics of all organisms in the system. Natural  
37 enemies also compete with one another, and the impact on the host can be either synergistic,  
38 additive, or antagonistic<sup>2</sup>. These interactions can be manifested in various aspects of host fitness  
39 or mortality. For example, biological control of *Drosophila suzukii* (Diptera, Drosophilidae), an  
40 important pest of fruit and berry crops, can be improved by treatments combining multiple  
41 natural enemies, which have an additive effect<sup>3</sup>. At the same time, dual infections (even if  
42 causing an increase in host mortality) may be deleterious to one or both pathogens in terms of  
43 pathogen growth, fecundity, or other fitness parameters.

44  
45 *Harmonia axyridis* (Coleoptera, Coccinellidae), native to eastern Asia, has rapidly increased its  
46 global range and is now present on all continents except Antarctica<sup>4,5,6</sup>. Even though it has  
47 repeatedly been introduced for its beneficial properties as a biological control agent against aphid  
48 pests, its negative effects on native ladybird communities in invaded areas<sup>7,8,9</sup> and on food  
49 production<sup>7</sup> have raised serious concerns since the early 2000s<sup>4</sup>. It is now a model organism for  
50 studying invasive alien species<sup>10,11</sup> and it has been listed in Europe as “one of the worst” invasive  
51 species<sup>12</sup>. *Harmonia axyridis* is often reported as host to several natural enemies. These include  
52 parasites (*Hesperomyces virescens*, *Coccipolipus hippodamiae*, *Parasitylenchus bifurcatus*),  
53 parasitoids (phorid and tachinid flies, *Dinocampus coccinellae*, *Homalotylus* spp., Tetrastichinae  
54 spp.), pathogens (bacteria, fungi, nematodes, protozoans), and predators (bugs, lacewings,  
55 ladybirds, and spiders)<sup>13,14,15,16,17,18</sup>. Independent studies show that natural enemies of native

56 ladybirds have recently employed *Ha. axyridis* as a new host, sometimes simultaneously<sup>16,17,19,20</sup>.  
57 Review of the effects of parasites, pathogens, and parasitoids of *Ha. axyridis* shows that they  
58 have only limited potential for controlling population densities of their host when acting  
59 alone<sup>14,17,19,21</sup>. Thus far, no studies have focused on the effects of infections of multiple natural  
60 enemy on *Ha. axyridis*.

61  
62 *Hesperomyces virescens* (Ascomycota, Laboulbeniomycetes, Laboulbeniales) is a common  
63 obligate ectoparasite of ladybirds<sup>4,17</sup>. Contrary to most multicellular fungi, *He. virescens* and  
64 other members of Laboulbeniales lack hyphae, instead they form 3-dimensional multicellular  
65 thalli by determinate growth<sup>17,22</sup>. Laboulbeniales, including *He. virescens*, cannot be grown in  
66 axenic culture and no asexual stages are known<sup>22</sup>. Given locally high prevalence of the fungus  
67 *Hesperomyces virescens* (Ascomycota, Laboulbeniomycetes, Laboulbeniales) on ladybird  
68 hosts<sup>17,23</sup> and the abundance of entomopathogenic fungal strains in the environment<sup>24</sup>, we  
69 examined mortality of native and invasive *He. virescens*-infected ladybirds exposed to either  
70 *Beauveria bassiana* or *Metarhizium anisopliae* (Ascomycota, Sordariomycetes, Hypocreales).  
71 Because *He. virescens* forms a branched, non-septate, rhizoidal haustorium<sup>25</sup> that penetrates the  
72 host's exoskeleton and makes contact with the body fluid for nutrient uptake, we hypothesized  
73 that high thallus densities with concomitant haustorial formation by *He. virescens* weaken host  
74 defenses, thus increasing the host's susceptibility to infection by other natural enemies. With this  
75 experiment, we assess how *He. virescens* affects ladybird survival when exposed to other natural  
76 enemies that alone have little impact on *Ha. axyridis* and compare results with a North  
77 American-native ladybird of similar body size, *Olla v-nigrum*.

78

## 79 **Results**

80 Our candidate models for both host species *Ha. axyridis* and *O. v-nigrum* were significantly  
81 better at explaining survival relative to chance variation (Chi-squared test,  $\chi^2 = 156.7$ ,  $P < 0.001$ ;  
82  $\chi^2 = 153.0$ ,  $P < 0.001$ , respectively). The overall model fit was high for both candidate models  
83 (*Ha. axyridis*: Nagelkerke's  $R^2 = 0.40$ ; *O. v-nigrum*: Nagelkerke's  $R^2 = 0.53$ ) suggesting the  
84 variance is well described by our applied models.

85  
86 We found a significant negative effect on ladybird survival of the *M. anisopliae* treatment on *He.*  
87 *virescens*-negative *Ha. axyridis* (Fig. 1, Table 1), whereas *B. bassiana* treatments did not affect  
88 the survival of *He. virescens*-negative individuals. Infection with *He. virescens* significantly  
89 affected *Ha. axyridis* survival over all treatments (Fig. 1, Table 1). However, there was no  
90 additional effect detectable among co-infection treatments for *He. virescens*-positive ladybirds.  
91 Each treatment applied to *O. v-nigrum* had a significantly negative effect on the survival for both  
92 *He. virescens*-negative and -positive ladybirds (Fig. 1, Table 2). Finally, we found an additional  
93 negative effect of all co-infection treatments on the survival of *He. virescens*-positive *O. v-*  
94 *nigrum* (Fig. 1, Table 2). These results suggest that there is no effect of dual infections on *Ha.*  
95 *axyridis*, whereas *O. v-nigrum* is highly affected by simultaneous exposure to both *He. virescens*  
96 and an entomopathogenic fungus. Percentages of ladybird mortality by treatment are also  
97 presented in tabulated form in Supplementary Table S1.

98  
99 When comparing the daily survival of *Ha. axyridis* and *O. v-nigrum*, no significant differences  
100 were found in *He. virescens*-positive only treatments. However, when co-infected *O. v-nigrum*

101 showed a significantly lower survival compared to *Ha. axyridis* for native and GHA *B. bassiana*  
102 strains (log rank test,  $P = 0.0014$  and  $P < 0.001$ , respectively; Fig. 2).

103

## 104 **Discussion**

105 Research on the additive effects of multiple natural enemies on a given host is rare, likely  
106 because of the complexity involved in designing robust bioassays that include all partners of the  
107 system. Combining the natural enemies *Orius insidiosus* (Hemiptera, Anthocoridae) and  
108 *Heterorhabditis bacteriophora* (Rhabditida, Heterorhabditidae) resulted in the largest decline in  
109 larvae of *Drosophila suzukii*<sup>3</sup>, which causes major economic losses to fruit crops in its invasive  
110 range, spanning North and South America and Europe<sup>26</sup>. The addition of *O. insidiosus* resulted in  
111 50% fewer *D. suzukii* larvae compared to treatment with only *H. bacteriophora*. *Plutella*  
112 *xylostella* (Lepidoptera, Plutellidae), an important cosmopolitan pest of brassicaceous crops,  
113 offers another example. This organism shows resistance to almost all chemical insecticides<sup>27</sup>.  
114 *Pandora blunckii* and *Zoophthora radicans* (Zoopagomycota, Entomophthoromycetes,  
115 Entomophthorales) both infect *P. xylostella* in the field. In co-inoculation studies with *Pa.*  
116 *blunckii* and *Z. radicans* in *P. xylostella* larvae, larval cadavers (three days post mortality) were  
117 most frequently found with conidia of a single entomopathogen, usually the one that had been  
118 inoculated first (prior “residency”)—meaning that the other species was excluded<sup>28</sup>. In general,  
119 the presence of competing species in the same host resulted in a decreased proportion of *P.*  
120 *xylostella* larvae that were infected compared to single inoculations.

121

122 Regarding *Ha. axyridis*, the following co-infections of natural enemies have been observed in  
123 nature: *He. virescens* + *Coccipolipus hippodamiae* mites (Acarina, Podapolipidae) in the USA,

124 Austria, and the Netherlands<sup>14,16,29</sup> and *He. virescens* + *Parasitylenchus bifurcatus* nematodes  
125 (Nematoda, Allantonematidae) in the Czech Republic, Germany, and the Netherlands<sup>16,17,30</sup>.  
126 Given the status of *Ha. axyridis* as an invasive alien species, these findings demand a better  
127 understanding of interactions among the different natural enemies and their potential role in  
128 limiting populations of *Ha. axyridis*. To date, bioassays to determine mortality of ladybirds  
129 induced by infection by one or more natural enemies have not yet been performed. Likewise,  
130 bioassays including Laboulbeniales have only been carried out in one study<sup>31</sup>.

131  
132 When we started this study, *He. virescens* was considered a single species with multiple ladybird  
133 hosts, potentially with multiple strains that infect only a single species, or one closely related<sup>32</sup>.  
134 Yet, it was recently shown that *He. virescens* is a complex of multiple species, each with its own  
135 ladybird host<sup>33</sup>. Isolates of *He. virescens* from *Ha. axyridis* and *O. v-nigrum* represent two  
136 different species of *Hesperomyces*. In other words, the experiments in the current study allow us  
137 to make comparisons between two host species, each with their own specific fungal parasite. To  
138 disentangle the interactions in future experiments, we must infect *Ha. axyridis* and *O. v-nigrum*  
139 ladybirds with the species of *Hesperomyces* specific to *Olla* and *Harmonia*, respectively,  
140 perform bioassays, and compare mortality rates under different treatments with our current  
141 results. Analyzing interactions among natural enemies only make sense when the taxa considered  
142 represent single biological species.

143  
144 We found a significant negative effect of *Hesperomyces*-only infection on the survival of both  
145 ladybird hosts (Fig. 1a, Supplementary Table S1). Previous work has shown *Hesperomyces*  
146 infections to result in decreased mating frequency of female ladybirds, lower (male) survival

147 rates in winter, and impeded sensing ability and flexibility of legs in heavily infected  
148 ladybirds<sup>14,17,34</sup>. One study<sup>35</sup> implicated parasitism by *He. virescens* as the cause of late summer  
149 mortality of *Chilocorus bipustulatus* ladybirds but this was later disputed based on controlled  
150 laboratory experiments<sup>36</sup>. Our research is the first to explicitly link *Hesperomyces* infection with  
151 increased ladybird mortality.

152  
153 Our findings provided a unique opportunity for our dual infection assays—the first such  
154 experiments to be conducted on ladybirds. When first infected with *He. virescens* and then  
155 exposed to either *B. bassiana* or *M. anisopliae*, *Ha. axyridis* mortality was not increased. This  
156 result was unexpected. We had hypothesized that *Ha. axyridis* with high thallus densities of *He.*  
157 *virescens* would have lowered host defenses against other pathogens. In contrast, the mechanism  
158 fostering low susceptibility of *Ha. axyridis* to entomopathogenic fungi<sup>20,37</sup> is not compromised  
159 by infection with *He. virescens*. Similarly, infection of *O. v-nigrum* by *He. virescens*-only  
160 increased mortality but—in contrast to *Ha. axyridis*—there was significantly higher mortality  
161 when co-infected by entomopathogenic fungi. Differential susceptibility to entomopathogenic  
162 fungi was reported by Cottrell and Shapiro-Ilan<sup>37</sup>, who showed that native *B. bassiana* was  
163 pathogenic to *O. v-nigrum* but not to *Ha. axyridis*. We confirm these results regarding the native  
164 strain but we also found the same differential pattern for the GHA strain of *B. bassiana*, whereas  
165 in the earlier study this strain was reported to be pathogenic to neither ladybird species<sup>37</sup>. It is  
166 perhaps surprising that we detect the GHA strain to be pathogenic to native ladybirds in contrast  
167 to the previous results, but ladybird populations may become more susceptible over time for  
168 various reasons and natural enemies also become better adapted<sup>20</sup>. We note that differential



169 susceptibility has also been reported for entomopathogenic nematodes—again, *Ha. axyridis* was  
170 less susceptible compared to *O. v-nigrum*<sup>38</sup>.

171  
172 In addition, our data are the first account of differential susceptibility to *M. anisopliae* between  
173 the invasive *Ha. axyridis* and the native *O. v-nigrum*. Whereas infection with *M. anisopliae* had a  
174 significantly negative effect on the survival of *He. virescens*-negative *Ha. axyridis*, this effect  
175 was not visible in the dual infection treatment. The infection with Laboulbeniales probably  
176 decreased the susceptibility of *Ha. axyridis* to infection by *M. anisopliae*, similar to the findings  
177 of Konrad and colleagues<sup>31</sup>. These authors found that *Laboulbenia*-infected *Lasius neglectus* ants  
178 (Hymenoptera, Formicidae) showed a decreased susceptibility to *Metarhizium brunneum*. This  
179 protection against *Metarhizium* was positively correlated with parasite load. Information on the  
180 parasite load of *He. virescens* on ladybirds in nature is scarce. In our bioassays, we selected  
181 ladybirds bearing 14 or more fungal thalli as *He. virescens*-positive specimens. Previous work  
182 from a long-term ATBI project in the Netherlands<sup>39</sup> points at an average of  $19.8 \pm 4.9$  thalli and a  
183 maximum of 129 thalli per *Ha. axyridis* specimen ( $n = 270$ ). No such data are available for *O. v-*  
184 *nigrum*. In other words, based on the available information, the artificial parasite load in our  
185 bioassays seems to closely mimic the natural conditions.

186  
187 Our results provide direct support for the enemy release hypothesis<sup>40</sup>. This hypothesis is  
188 illustrative for the success of invasive alien species and stipulates that an invasive species in new  
189 geographic regions will experience reduced regulatory effects from natural enemies compared to  
190 native species, resulting in increased population growth of the invasive species<sup>41,42</sup>. However,  
191 invasions are dynamic<sup>17,42</sup> and this escape-from-enemies could be lost as invasive species acquire

192 new enemies over time<sup>44</sup>. Support for enemy release explaining the success of *Ha. axyridis* has  
193 come from two studies that reported decreased susceptibility of *Ha. axyridis* to  
194 entomopathogenic fungi<sup>37</sup> and entomopathogenic nematodes<sup>38</sup> compared to the native American  
195 ladybird species. Our work adds another level of complexity by the addition of a second natural  
196 enemy to the interactions. Again, we find differential susceptibility between the invasive and  
197 native ladybird species—with a reduced regulatory effect of the tested natural enemies on *Ha.*  
198 *axyridis*.

199  
200 In this paper, we show a negative effect of infection by *Hesperomyces virescens* on the survival  
201 of both *Harmonia axyridis* and *Olla v-nigrum* ladybirds (Fig. 1a). This is the first study to  
202 unequivocally link *Hesperomyces* infection with increased host mortality and only the second to  
203 perform bioassays with hosts co-infected with Laboulbeniales and a second entomopathogenic  
204 fungus<sup>31</sup>. However, the susceptibility to a secondary entomopathogenic fungus was only elevated  
205 in the native American ladybird species (*O. v-nigrum*), whereas the globally invasive *Ha.*  
206 *axyridis* showed no significant increase in mortality when co-infected with either *Beauveria*  
207 *bassiana* or *Metarhizium anisopliae* (Figs. 1, 2). These findings are consistent with the enemy  
208 release hypothesis<sup>38</sup> and highlight the difficulty in controlling this invasive alien species. Future  
209 studies are needed to elaborate population-specific effects on native and commercial strains of  
210 entomopathogenic fungi used in pest control.

211

## 212 **Methods**

213 *Field collections and laboratory colonies*

214 *Harmonia axyridis* and *Olla v-nigrum* ladybirds were collected for the purpose of establishing  
215 laboratory colonies of *Hesperomyces*-infected and non-infected ladybirds. Specimens were  
216 collected at overwintering sites at the 485-ha USDA-ARS, Southeastern Fruit and Tree Nut  
217 Research Laboratory, located in Byron, Georgia, USA (32.657792, -83.7383580). Sex and age of  
218 field-collected specimens were not determined to reduce dispersal of fungal propagules<sup>32</sup>. All  
219 specimens were brought to the laboratory and housed in individual Petri plates (10 cm diam.)  
220 with 1/3 of a piece of a cotton dental wick (Deerpack Products, LLC, Miami, Florida) drenched  
221 in water for hydration. Ladybirds were housed in environmental chambers at  $25 \pm 1$  °C and  
222 photoperiod of 14:10 (L:D) h. Food was provided 3× per week in the form of *Ephestia*  
223 *kuehniella* eggs (Lepidoptera, Pyralidae) and an artificial meat-based diet (Beneficial Insectary,  
224 Redding, California). *Olla v-nigrum* and *Ha. axyridis* ladybirds were maintained within the Petri  
225 plates for 14d and 21d<sup>32</sup>, respectively, at which time ladybirds were visually examined for  
226 presence of *Hesperomyces* using a dissecting microscope at 50× magnification. Eggs were  
227 harvested from ovipositing ladybirds and used to establish clean (free from fungal growth)  
228 laboratory-reared colonies of ladybirds with known age.

229

### 230 *Laboratory rearing of ladybirds*

231 During examination for presence/absence of *Hesperomyces*, ladybirds were divided into two  
232 groups, infected and non-infected. Both groups of ladybirds were placed into plastic rearing  
233 containers of 19 × 13.5 × 9 cm (Pioneer Plastics, North Dixon, Kentucky), which were modified  
234 with two 3-cm diameter circular openings, one that was covered by 1 × 1 mm mesh to allow for  
235 air flow; and the second that was covered with a removable #7 rubber stopper to allow for  
236 feeding routinely as well as adding newly emerged laboratory-reared ladybirds. Routine

237 maintenance included transferring ladybirds into fresh rearing containers at the end of each 7d  
238 period, which included nutrient supplementations of laboratory-reared yellow pecan aphids,  
239 *Monelliopsis pecanis* (Hemiptera, Aphididae).

240  
241 The first laboratory generation of adults emerged about one month after placement in rearing  
242 containers. Emerging adults were placed into fresh rearing containers and stored into a separate  
243 incubator ( $25 \pm 1$  °C, 14:10 (L:D) h) for 7 days. Similar to field-captured *O. v-nigrum* and *Ha.*  
244 *axyridis*, *M. pecanis* aphids were used as a diet augmentation. As the study progressed, we also  
245 incorporated black pecan aphids, *Melanocallis caryaefoliae* (Hemiptera, Aphididae), in the  
246 ladybird diet (3× per week).

247  
248 *Artificial transmissions of Hesperomyces*

249 Exposure to *Hesperomyces* was conducted via tumbling of the field-captured ‘source’ ladybirds  
250 (infected with *Hesperomyces*) with randomly selected laboratory-reared ‘target’ ladybirds<sup>45</sup>. A  
251 total of 25 target ladybirds were mixed with 5 *Hesperomyces*-infected source ladybirds in a 1.6 ×  
252 5.8 cm glass tube, which was placed on a hot-dog roller (Nostalgia Electrics, Green Bay,  
253 Wisconsin) for 5 min. This procedure was repeated for at least 160 target ladybirds of both  
254 species. Both *Hesperomyces*-exposed target ladybirds and clean (unexposed) ladybirds were fed  
255 a diet of *M. pecanis* aphids for 24h. We did a second tumbling experiment using randomly  
256 selected emerged adults from the second cohort of laboratory-reared colonies. More tumbling  
257 experiments were performed to increase quantities of *Hesperomyces*-infected ladybirds, but  
258 source/target numbers were changed to 100/40.

259

260 To reduce competition for food, ladybirds from all laboratory colonies were transferred from the  
261 plastic rearing containers to 14-cm diameter Petri plates. Ladybirds were provided with water ad  
262 libitum, *E. kuehniella* eggs, and artificial meat-based diet. Finally, for assay preparation, the  
263 ladybirds were transferred back to clean  $19 \times 13.5 \times 9$  cm plastic rearing containers.

264

#### 265 *Dual fungal infections assay*

266 Within 24 hours preceding the assay, 160 non-infected and 160 *Hesperomyces*-infected ladybirds  
267 of each species (*Ha. axyridis* and *O. v-nigrum*) were each placed into sterile test tubes, which  
268 were then closed with a sterile foam stopper to prevent ladybirds from escaping while allowing  
269 for air flow. Infected ladybirds were divided into categories according to numbers of thalli per  
270 specimen. Because the assay would assess potential interactions between fungal infections, we  
271 aimed at selecting heavily *Hesperomyces*-infected ladybirds; as a baseline, we only used  
272 specimens in our bioassays with 14 or more thalli each.

273

274 The assay started by pipetting a 1 mL of  $2.5 \times 10^5$  conidia/mL suspension to each test tube<sup>37,45</sup>.  
275 Treatments included native *B. bassiana* (native Bb), a commercial *B. bassiana* strain (GHA Bb;  
276 Mycotrol ES, Mycotech, Butte, Montana), *M. anisopliae* strain 52 (Ma, isolated from a tortricid  
277 moth, Austria 1971; Novozymes, Franklinton, North Carolina), and double-distilled water  
278 (ddH<sub>2</sub>O) as a control treatment. Ladybirds were submerged and swirled for 5 s, after which the  
279 suspension was removed again using a pipette and each ladybird was placed into a 6 cm-  
280 diameter Petri plate. Any remaining droplets of excess suspension was removed by touching only  
281 the droplet with a Kimwipe tissue (Kimtech Science Brand, Kimberly-Clark Worldwide,  
282 Roswell, Georgia). Petri plates with treated ladybirds were placed into an incubator ( $25 \pm 1$  °C,

283 14:10 (L:D) h). Food and cotton rolls drenched in water were provided ad libitum, and Petri  
284 plates were replaced as needed in all treatments and replications simultaneously. Ladybirds were  
285 observed for mortality and entomopathogen-induced mycosis at day 14. During assay #1, we  
286 made daily observations for ladybird mortality and mycosis. Upon death of a given ladybird,  
287 ample water was added to the cotton roll to provide moisture for entomopathogen growth and  
288 Parafilm was applied around the Petri plate to prevent spreading of the fungus. Deaths of  
289 ladybirds and visual confirmations of mycosis were recorded.

290

291 We performed 8 different treatments for each ladybird species: 1) *He. virescens*-positive + native  
292 Bb, 2) *He. virescens*-positive + GHA Bb, 3) *He. virescens*-positive + Ma, 4) *He. virescens*-  
293 positive + ddH<sub>2</sub>O (control), 5) *He. virescens*-negative + native Bb, 6) *He. virescens*-negative +  
294 GHA Bb, 7) *He. virescens*-negative + Ma, and 8) *He. virescens*-negative + ddH<sub>2</sub>O (double  
295 control). In a single assay, we replicated every treatment 3 or 4 times. We performed the entire  
296 assay with all treatments and replicated 3 times, using 6–10 ladybirds for each treatment. Note  
297 that *M. anisopliae* treatments were used only in assay #3 (Supplementary Table S2). Over all  
298 assays done during this study, we used 1,289 specimens of ladybirds (667 *O. v-nigrum* and 622  
299 *Ha. axyridis*).

300

### 301 *Statistical analyses*

302 All statistical analyses were performed in the R language and open-access environment for  
303 statistical computing v.3.5.0. We used generalized linear mixed models (function `glmer()`, R-  
304 package *lme4*<sup>46</sup>) to analyze the effect of the different treatments (GHA Bb, native Bb, Ma) on the  
305 survival of *Ha. axyridis* and *O. v-nigrum* in relation to prior infection with *Hesperomyces*. We

306 modeled the binary response variable survival (alive/dead) of each ladybird individual for both  
307 host species separately, and used *Hesperomyces* infection status as well as the interaction of  
308 *Hesperomyces* infection status with treatment as explaining variables. Further, to correct for  
309 variation within replicates and trials, we included the random effect of treatment nested in  
310 replicate nested in trial. We compared our candidate models to a respective Null-model using  
311 likelihood ratio tests and, furthermore, calculated pseudo  $R^2$ -values (function `r2()`, R package  
312 *sjstats*<sup>47</sup>) to evaluate model fit. To visualize the modeling results and obtained model estimates as  
313 forest plots, we used the function `plot_model()` implemented in the R package *sjstats*<sup>47</sup>. For assay  
314 #1, we further fitted Kaplan-Meier curves to daily mortality data and tested for significant  
315 differences in mortality between ladybird species using the function `survfit()` of the R package  
316 *survival*<sup>48</sup>.

317

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456

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464 **Author contributions**

465 Conceptualization and methodology: D.H., T.E.C. Investigation: D.H., E.A.K., T.E.C. Data  
466 curation: D.H., T.H., P.S.v.W., T.E.C. Formal analysis: D.H., T.H., P.S.v.W. Resources,  
467 supervision: D.I.S.I., M.C.A., O.N., D.H.P., T.E.C. Visualization: D.H., T.H. Writing: D.H.,  
468 T.H., E.A.K. with input from the other co-authors.

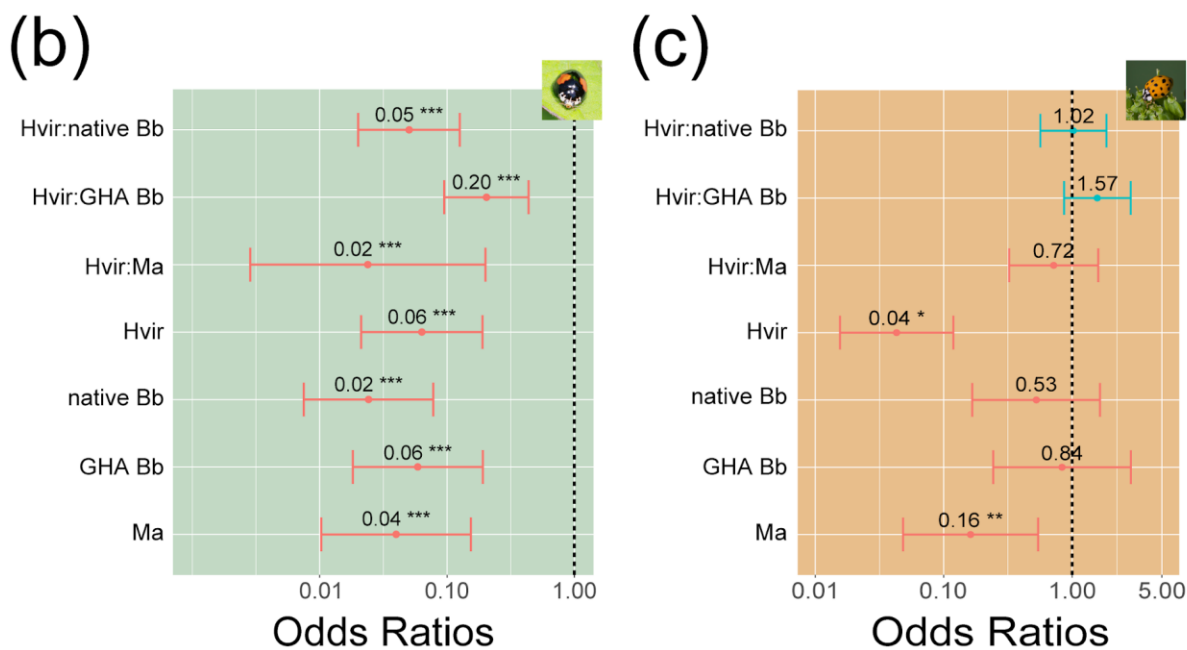
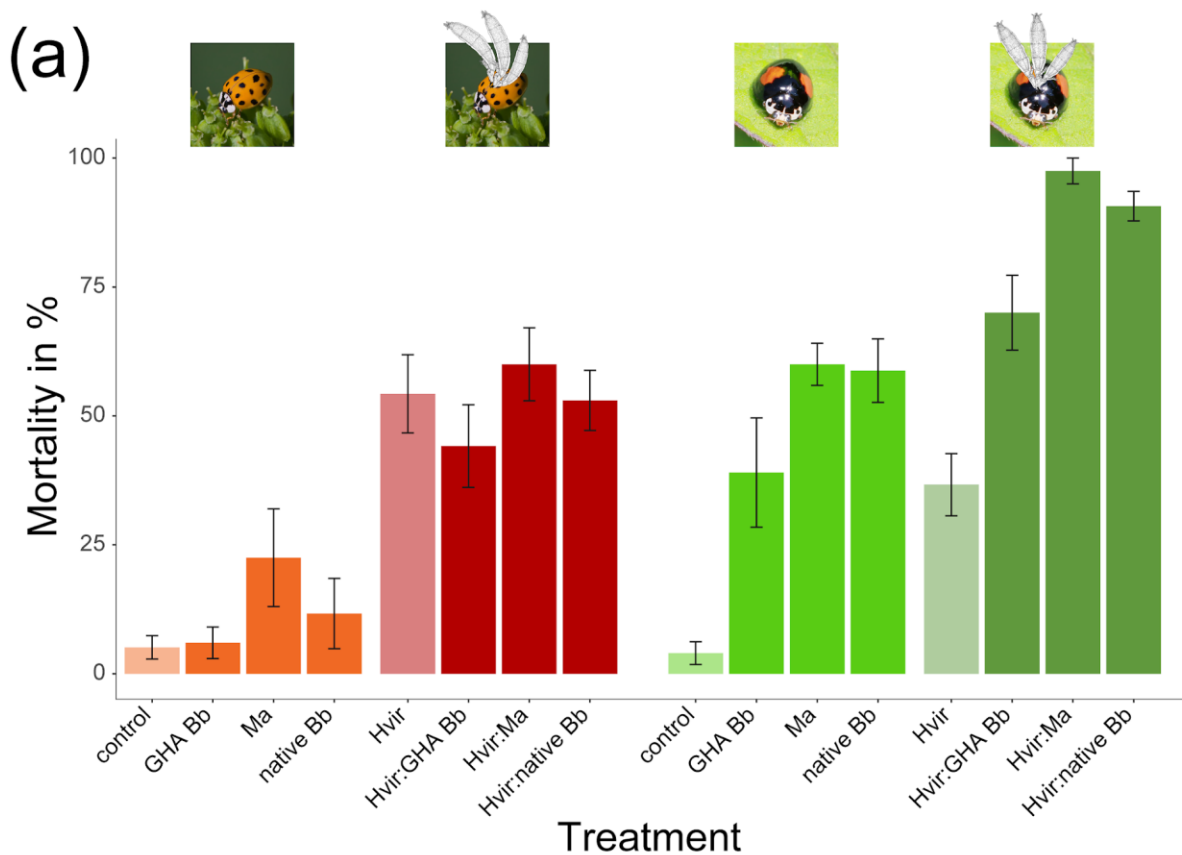
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470 **Competing interests**

471 The authors declare that they have no competing interests.

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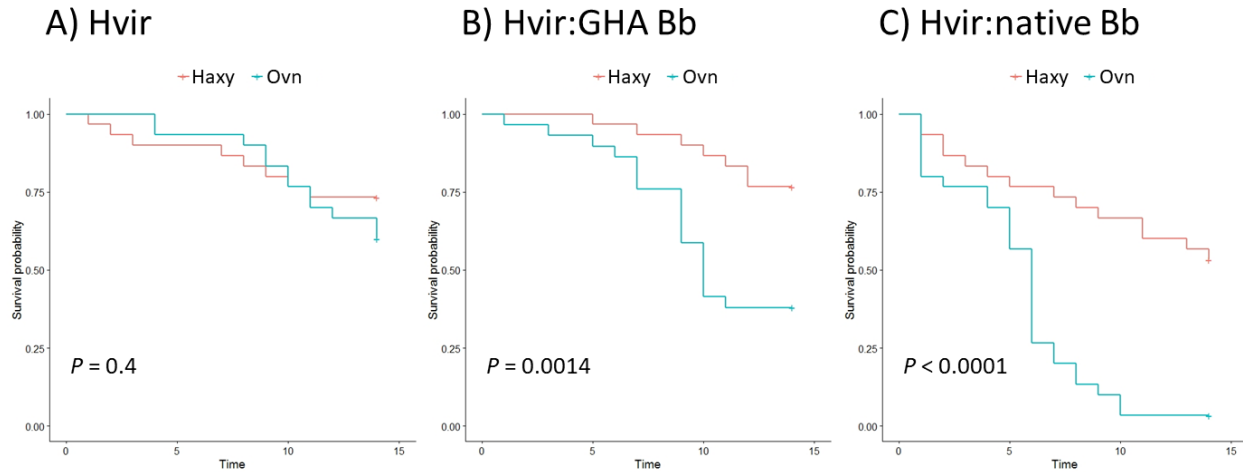
473 **Figures & tables**



474

475 **Figure 1: Results of treatment effects on native and invasive ladybirds.**

476 **A.** Percentages of ladybird mortality by treatment (left *Harmonia axyridis*, right *Olla v-nigrum*).  
477 **B–C.** Forest plots illustrating the results of our modelling approach showing the treatment effects  
478 on survival of ladybirds (negative effect in red (odds ratio < 1), positive effect in blue (odds ratio  
479 > 1); \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ). **B.** *Olla v-nigrum*. **C.** *Harmonia axyridis*. Photo credits: *Olla*  
480 *v-nigrum*, Roberto Güller (Flickr); *Harmonia axyridis*, Andreas Trepte (Wikimedia Commons).  
481 Drawings of *Hesperomyces thalli* by André De Kesel.  
482  
483



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485 **Figure 2: Survival of ladybirds under different treatments.**

486 **A.** Survival of *Hesperomyces*-positive ladybirds without dual infection. **B.** *Hesperomyces*-  
487 positive ladybirds co-infected with GHA strain of *Beauveria bassiana*. **C.** *Hesperomyces*-  
488 positive ladybirds infected with native *Beauveria bassiana*. The survival of ladybirds is  
489 significantly different between *Harmonia axyridis* and *Olla v-nigrum* with the dual infection  
490 treatments.

491



**Table 1. Results of our modeling approach for *Harmonia axyridis*.**

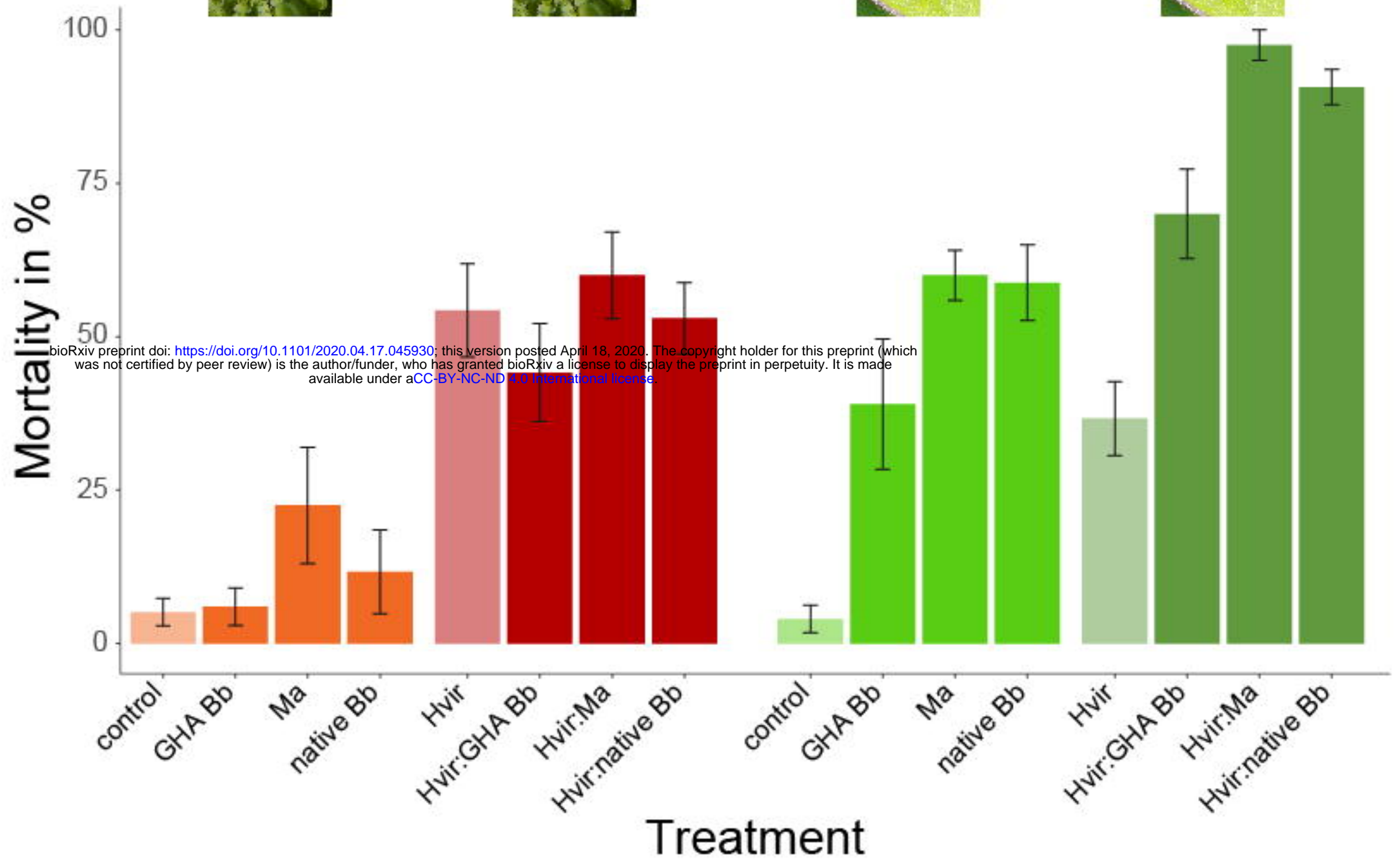
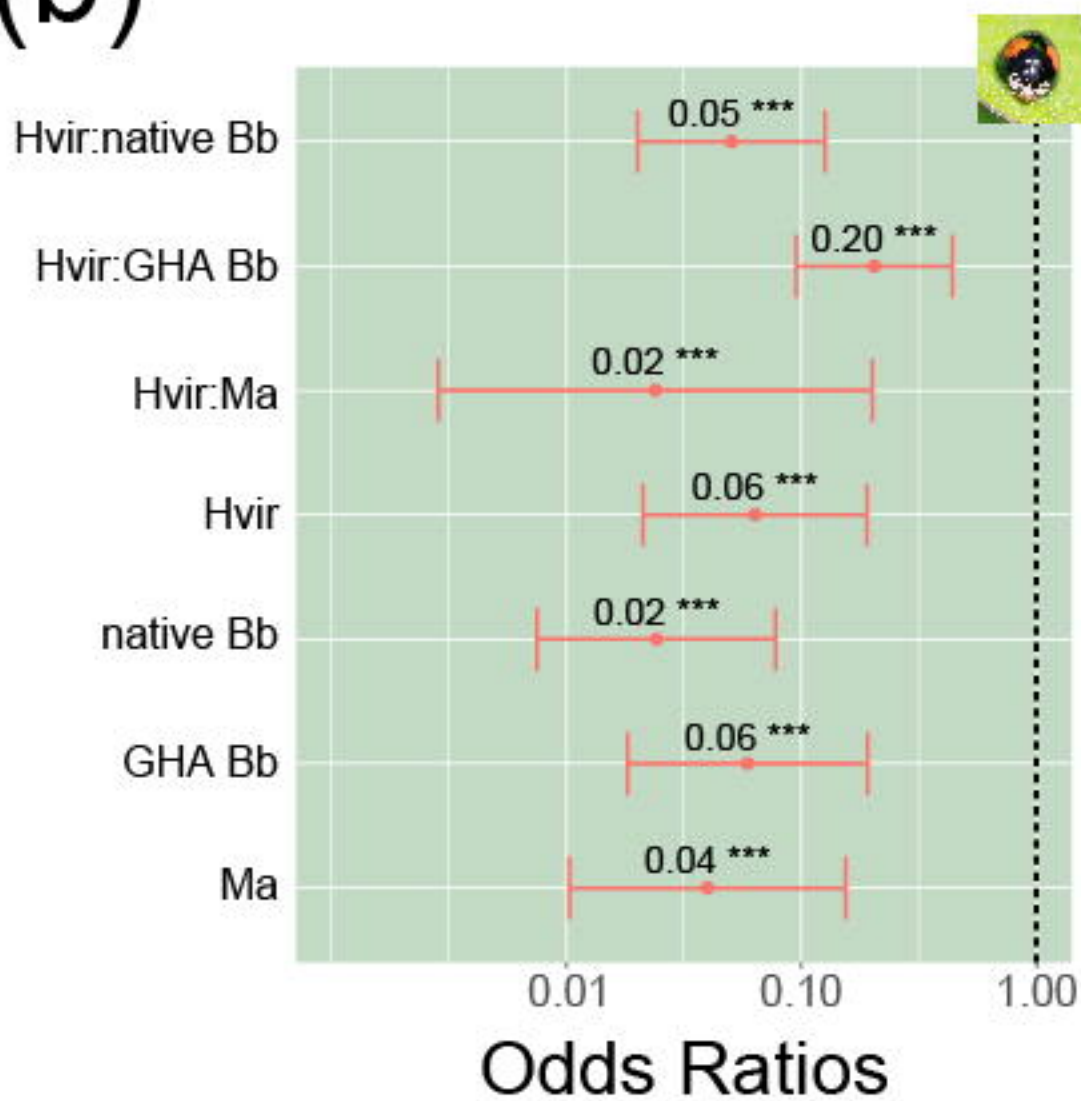
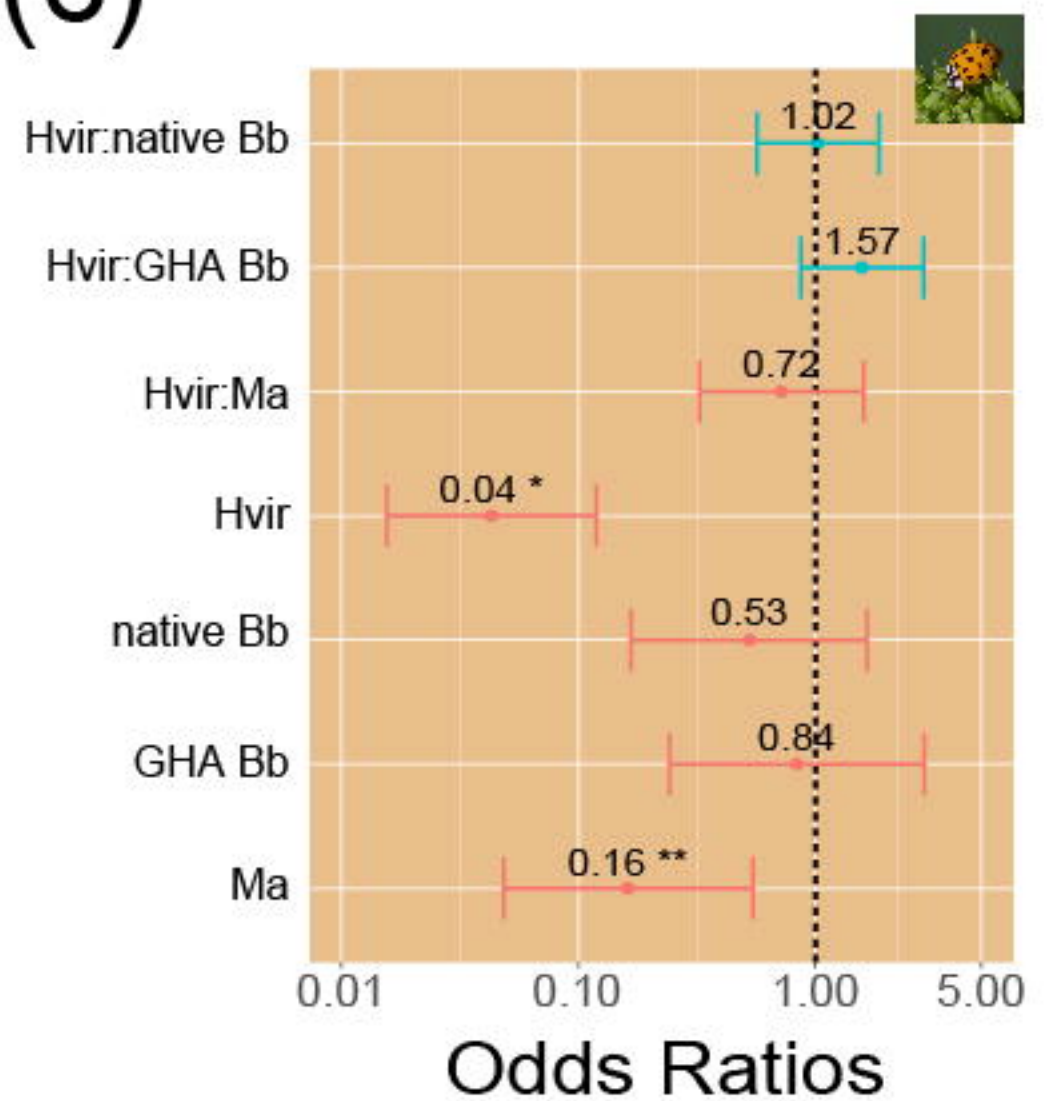
Summary of parameters corresponding to *Hesperomyces virescens* infection and respective interactions with GHA *Beauveria bassiana*, native *B. bassiana*, and *Metarhizium anisopliae*.

		Estimate	Std. Error	z value	P-value
(Intercept)		2.977	0.563	5.284	< <b>0.001</b> ***
<i>He. virescens</i> infection		-3.147	0.518	-6.071	< <b>0.001</b> ***
<i>He. virescens</i> -negative	GHA Bb	-0.179	0.630	-0.284	0.777
	Native Bb	-0.642	0.585	-1.098	0.272
	Ma	-1.820	0.618	-2.946	<b>0.003</b> **
<i>He. virescens</i> -positive	GHA Bb	0.454	0.305	1.487	0.137
	Native Bb	0.024	0.302	0.080	0.936
	Ma	-0.330	0.406	-0.811	0.417

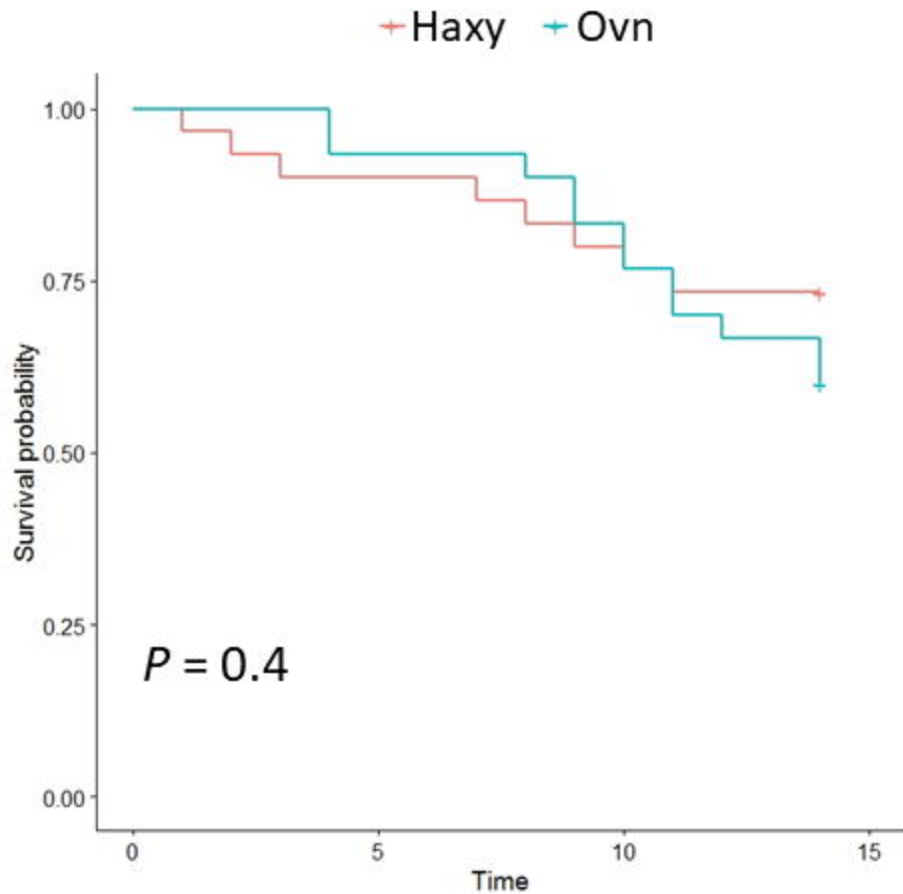
**Table 2. Results of our modeling approach for *Olla v-nigrum*.**

Summary of parameters corresponding to *Hesperomyces virescens* infection and respective interactions with GHA *Beauveria bassiana*, native *B. bassiana*, and *Metarhizium anisopliae*.

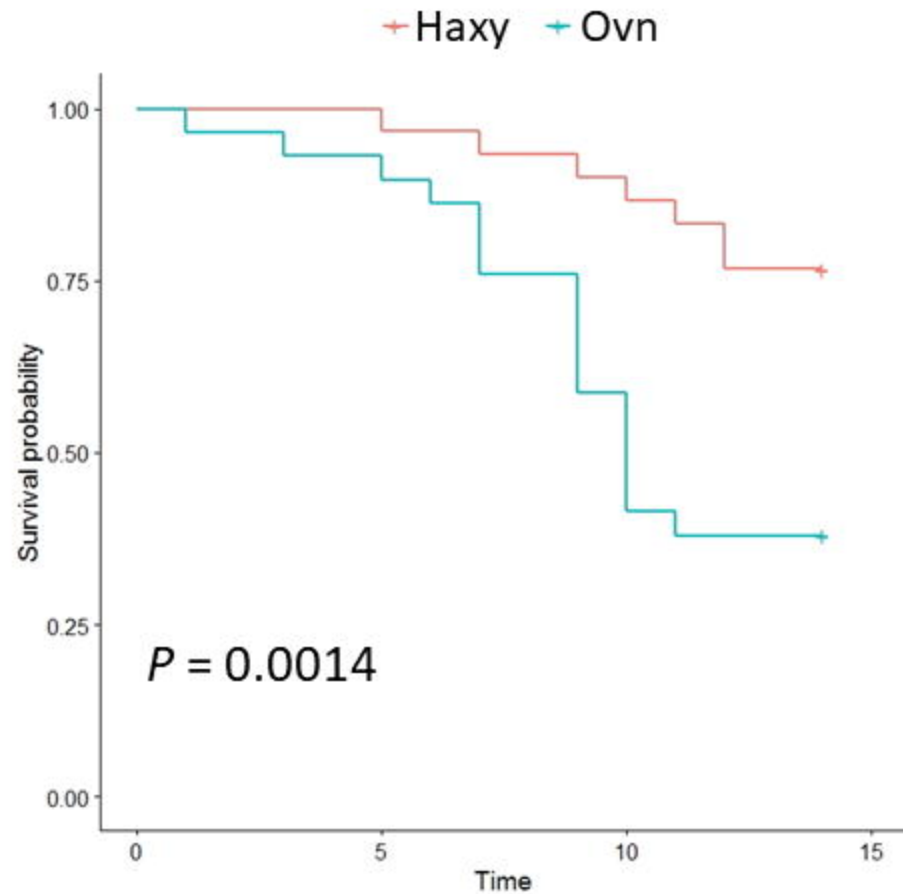
		Estimate	Std. Error	z value	P-value
(Intercept)		3.407	0.609	5.598	< <b>0.001</b> ***
<i>He. virescens</i> infection		-2.757	0.560	-4.925	< <b>0.001</b> ***
<i>He. virescens</i> -negative	GHA Bb	-2.831	0.599	-4.728	< <b>0.001</b> ***
	Native Bb	-3.723	0.597	-6.234	< <b>0.001</b> ***
	Ma	-3.222	0.689	-4.676	< <b>0.001</b> ***
<i>He. virescens</i> -positive	GHA Bb	-1.591	0.390	-4.084	< <b>0.001</b> ***
	Native Bb	-2.988	0.470	-6.364	< <b>0.001</b> ***
	Ma	-3.734	1.084	-3.444	< <b>0.001</b> ***

**(a)****(b)****(c)**

# A) Hvir



# B) Hvir:GHA Bb



# C) Hvir:native Bb

