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**Invasive *Vespa velutina nigrithorax* hornets are more susceptible to entomopathogenic fungus than two other hymenopteran species, the wasp *Vespa vulgaris* and the bumblebee *Bombus terrestris* .**

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1 **Invasive *Vespa velutina nigrithorax* hornets are more susceptible to**  
2 **entomopathogenic fungus than two other hymenopteran species, the wasp**  
3 ***Vespa vulgaris* and the bumblebee *Bombus terrestris*.**

4

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## 19 **Abstract**

20 Entomopathogenic fungi are commonly used as biological control agents. Recently, a strain of  
21 *Metarhizium robertsii* (Sorokin, 1883) (Hypocreales: Clavicipitaceae) collected in France was  
22 identified as a good candidate for controlling invasive populations of yellow-legged hornet, *Vespa*  
23 *velutina nigrithorax* (Buysson, 1905) (Hymenoptera: Vespidae) in Europe. Since *M. robertsii* is  
24 generalist, its use as bioagent could be damaging for non-targeted species. Here, we compared the  
25 lethal effects of three concentrations of this *M. robertsii* strain on the invasive hornet and two non-  
26 targeted species commonly found in Europe, the common wasp *Vespa vulgaris* (Linnaeus, 1758)  
27 (Hymenoptera: Vespidae) and the buff-tailed bumblebee *Bombus terrestris* (Linnaeus, 1758)  
28 (Hymenoptera: Apidae). Exposure to *M. robertsii* spores altered the survival of all three species but at  
29 different degrees. Hornets had consistently lower survival, even under low concentrations of spores,  
30 when compared to wasps and bumblebees. Such lower susceptibility of beneficial Hymenoptera is  
31 encouraging in the perspectives of using *M. robertsii* as biocontrol agent against invasive hornets.

32

33 **Keywords:** *Hymenoptera*, *Vespidae*, *Hypocreales*, *Clavicipitaceae*, biological control, invasive  
34 species

35

## 36 **Statements and Declarations**

37 The authors declare no competing interests. This study was funded by the ADEME (No. 2082C0061)  
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40

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## 43 **Introduction**

44 The yellow-legged hornet (*Vespa velutina nigrithorax*) (Buysson, 1905) (Hymenoptera: Vespidae) is  
45 an invasive social insect species throughout the world, especially in Europe (Haxaire et al. 2006),  
46 Korea (Kim et al. 2006) and Japan (Sakai and Takahashi 2014) (Thiery and Monceau 2024). Their  
47 geographic expansion poses major concerns to beekeeping activities and the environment (Monceau  
48 et al, 2014). Current methodologies to control hornet populations are expensive and use pesticides  
49 harmful for the biodiversity (Kishi and Goka 2017; Goulson 2019). Recently, however, several  
50 biocontrol options have been explored (Turchi and Derijard 2018) including the use of an  
51 entomopathogenic fungus, *Metarhizium robertsii* (Sorokin, 1883) (Hypocreales: Clavicipitaceae)  
52 (Poidatz et al. 2018). This fungus pertains to the complex of species *M. anisopliae* that is already  
53 commercialized for biocontrol and known to be efficient against Orthopterans, Isopterans and  
54 Thysanopterans (i.e. *M. anisopliae* strain F52 approved in Europe, USA, Brazil, Columbia and China  
55 for the use against various insects; *M. anisopliae* strain FI-985 approved in Australia, India and China  
56 for the use against grasshoppers and locusts). In particular, Poidatz et al. (2018) have demonstrated  
57 that the exposure to spores of four isolates of *M. robertsii* (EF2.5(1), EF3.5(1), EF3.5(2), EF3.5(4)) at  
58  $10^7$  sp/ml reduced by half the survival of yellow-legged hornets after only 5 to 6 days.

59  
60 From a biological control perspective, entomopathogenic fungi like *M. robertsii* constitute good  
61 candidates to target a social insect such as hornets. On the one hand, sociality favors parasite  
62 transmission between genetically close individuals that are spatially aggregated, and engaged in  
63 numerous behavioral interactions, through social contacts, food exchange, or proximity with nest  
64 materials (Dimbi et al. 2013; Khun et al. 2021). However, the potential infection of non-targeted  
65 species should also carefully be taken into consideration if infected hornets leave their nest and fly,  
66 disseminating spores from their body surface (Smaghe et al. 2013). In particular, there is a risk that  
67 wild and managed pollinators get contaminated through direct contact with spores when contaminated  
68 hornets hunt unsuccessfully and release their prey (Ueno 2015), or land on a honeybee hive flight  
69 board, or contaminate flowers on which they collect nectar (Kapongo et al. 2008).

70 Several studies on the complex species of *M. anisopliae* (including *M. robertsii*) (Rehner and Kepler  
71 2017) have reported negligible (Smagghe et al. 2013) to strong negative impact (Shaw et al. 2002) on  
72 several beneficial insects such as pollinators (e.g. honey bees, bumblebees), depending on the doses  
73 (Kanga et al. 2003; Smagghe et al. 2013), the formula (Ball et al. 1994), and the methods of  
74 application used (Rodríguez et al. 2009). For instance, the contact with a powder concentrate of  $10^7$   
75 sp/g decrease the survival of bumblebees by almost 25% within 6 weeks (Smagghe et al. 2013). The  
76 decrease of survival reaches 100% at a concentration of  $10^9$  sp/g. The survival of honey bees was  
77 lower when the fungus was sprinkled on and between the frames of a hive (decreased by around 55%)  
78 than when conidia were stamped on filter papers and placed on half the frames (decreased by 5%)  
79 (Rodríguez et al. 2009). Given these potential advantages and disadvantages for hornet biocontrol,  
80 there is a need to better characterize the impact of *M. robertsii* exposure with comparable  
81 methodologies both on invasive hornets and non-target species before it can be further considered for  
82 biocontrol.

83

84 Here we compared the lethal effect of a *M. robertsii* strain EF3.5(1) on *V. v. nigrithorax* and two non-  
85 target pollinator species commonly found in the invaded area, the common wasp *Vespula vulgaris*  
86 (Linnaeus, 1758) (Hymenoptera: Vespidae) and the buff-tailed bumblebee *Bombus terrestris*  
87 (Linnaeus, 1758) (Hymenoptera: Apidae). We hypothesized that *M. robertsii* would have different  
88 effects depending on the ecology and the natural history of the host species. Specifically, we expected  
89 that the virulence of the parasite would be stronger in hornets than in the two other species, because  
90 these invasive insects only build aerial nests which could greatly limit the exposition to *M. robertsii*,  
91 whereas the local non-target species may have co-evolved with it since long by nesting in soil. We  
92 tested this hypothesis by exposing hornets, wasps, and bumblebees to different concentrations of *M.*  
93 *robertsii* and monitoring their survival for eight days.

94

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96

## 97 **Methods**

### 98 *Insects*

99 All the experiments were performed in Bordeaux (France) during summers 2021 to 2023. We used  
100 four wild *V. v. nigrithorax* nests, one wild *V. vulgaris* nest, and four commercial *B. terrestris* nests  
101 (see Table 1). Although commercially reared since about 30 years, these bumblebee populations  
102 originate from wild populations sampled in Europe and are frequently mixed with freshly caught wild  
103 individuals (Velthuis and Doorn 2005). For each species, we collected the adults from the different  
104 nests and grouped them into microcolonies of  $10 \pm 3$  individuals in plastic boxes (72 x 26.5 x 25 cm).  
105 Each microcolony was provided sucrose solution (40% v/v) and water *ad libitum*. Bumblebee  
106 microcolonies were also supplied with a commercial mixture of pollen (Icko-apiculture company,  
107 France).

### 108 *Entomopathogen fungus*

109 We used the strain EF3.5(1) of *M. robertsii* sampled in 2015 in an experimental INRAe vineyard  
110 (Villenave d'Ornon, France) (see Poidatz et al. 2018 for details). We selected this particular isolate  
111 because of its high virulence against hornets as shown by the direct inoculation and inoculation by  
112 transfer between hornets (Poidatz et al. 2018). *M. robertsii* was cultivated on a Petri dish (9 cm  
113 diameter) containing oat agar media (Oat flour 40g and Potatoes Dextrose Agar (Sigma Aldrich),  
114 Chloramphenicol (Sigma Aldrich), and permuted water) in a dark room. The fungus was transplanted  
115 every 2-3 months to ensure its healthy growth through nutrient resupplying.

### 116 *Infections*

117 We prepared spore solutions by mixing *M. robertsii* spores (extracted from culture media), distilled  
118 water, and 0.02% Tween80 (Sigma Aldrich) (Poidatz et al. 2018; Ponchon et al. 2022). The  
119 concentration of the solution was adjusted at  $10^3$  spores/ml (identified in a preliminary experiment as  
120 the sublethal concentration for bumblebees, named *low concentration*) or  $10^7$  spores/ml (named *high*

121 *concentration*) by counting spores in 10µl using a Neubauer cell. The control treatment was composed  
122 of distilled water and 0.02% Tween80. Each insect was exposed to one of these treatments by  
123 immersion of their entire body in the solution for 1 second. We used *M. robertsii* exposure by  
124 immersion because of its higher efficacy on hornet survival compared to exposure by contact (infected  
125 support or individual) or by food (Poidatz et al. 2018).

#### 126 *Survival assays*

127 We started the survival survey immediately after exposure to spores and the formation of  
128 microcolonies. From then on, we recorded the number of daily-dead insects and removed them from  
129 each microcolony for 8 consecutive days. We also monitored food consumption by weighing the  
130 containers of aqueous solutions (sucrose solution and water) every day.

#### 131 *Statistical analysis*

132 We ran all the analyses in R 4.3.3. We measured the effect of *M. robertsii* spore concentrations on  
133 survival for each species using a mixed-effect Cox model (*coxme* function, *survival* package,  
134 Therneau 2023) with treatment as a fixed factor and microcolony as a random effect. We ran a Cox  
135 proportional hazards regression model (*coxph* function, *survival* package, Therneau 2023) with the  
136 treatment as a fixed factor for wasps as they came from a single mother nest. We measured species  
137 sensibility differences by comparing the survival of species when exposed to a similar treatment. We  
138 used a mixed Cox model with species as a fixed factor and microcolony as a random factor. We  
139 calculated an estimate of the survival curve for each treatment within species using the *survfit*  
140 function (*survival* package, Therneau 2023) with the treatment as a fixed factor.

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## 145 **Results**

146 **Hornets were the most impacted by spore exposure.** We first tested the influence of *M. robertsii*  
147 concentrations on the survival of hosts (Fig. 1). For all three species, individuals exposed to high  
148 concentrations of spores showed reduced survival compared to unexposed controls of the same  
149 species (see Table 2 for statistics). Lowly-exposed hornets showed a significantly decreased survival  
150 by 3.16 times (Cox, 95% confidence interval [CI]: 1.21-8.24,  $p=0.048$ ) compared to controls. By  
151 contrast, in bumblebees, there was no difference between lowly-exposed individuals and controls  
152 (Table 2). Highly-exposed hornets showed a decreased survival by 36.83 times (Cox, CI: 14.58-93.08,  
153  $p<0.001$ ) compared to controls. This reduction was much higher than the reduction by 6.43 times  
154 (Cox, CI: 3.32-12.45) observed in wasps or by 1.61 times (Cox, CI: 0.76-3.44) in bumblebees at the  
155 same spore concentration. Overall, the difference in survival between exposure doses was larger in  
156 hornets than in wasps and in bumblebees (ANOVA, hornets: Treatment:  $X^2=73.43$ ,  $df=2$ ,  $p<0.001$ ;  
157 wasps: Treatment:  $X^2=40.67$ ,  $df=1$ ,  $p<0.001$ ; bumblebees: Treatment:  $X^2=7.88$ ,  $df=2$ ,  $p=0.019$ )  
158 indicating that non-targeted species (wasps and bumblebees) were less susceptible to spore exposure  
159 than hornets.

160

161 **Hornets and wasps lived longer than bumblebees at all spore concentrations.** We then compared  
162 the effects of the parasite on host survival between species and for each treatment. In unexposed  
163 controls, hornets and wasps showed a similar lifespan (Table 3 for statistics) and had a higher  
164 probability of survival than bumblebees (Table 3). When they were exposed to the parasite at low or  
165 high concentrations, hornets, and wasps also lived longer than bumblebees. At high concentrations,  
166 wasps survived longer than hornets (Table 3).

167



## 168 **Discussion**

169 We exposed three Hymenoptera species to different concentrations of a strain of entomopathogenic  
170 fungus recently reported to affect invasive yellow-legged hornets (Poidatz et al. 2018). Overall, for all  
171 spore concentrations tested, hornets were more susceptible to spore exposure than wasps and  
172 bumblebees, comforting the idea that *M. robertsii* is a good candidate for biocontrol against yellow-  
173 legged hornets with negligible impact on non-targeted species.

174

175 Our parasite strain EF3.5(1) harmed differently the survival of the three insect species depending on  
176 the exposure concentration. In particular, bumblebees had a shorter lifespan than the two other species  
177 but seemed less sensitive to entomopathogenic concentrations than the hornets. Since *B. terrestris*  
178 nests are found underground (Meyling and Eilenberg 2007), it is possible that they have already been  
179 in contact with closely related strains of *M. robertsii* during their evolution. Cases of natural exposure  
180 of bumblebees to *Metarhizium spp.* have already been recorded (Macfarlane et al. 1995; Schmid-  
181 Hempel 2001). In the context of host-parasite coevolution, bumblebees could have developed greater  
182 resistance to this parasite (Béréños et al. 2009) which should less (or not) be the case for yellow-  
183 legged hornets (invasive species, aerial niche) and wasps (local species but subterranean, or aerial  
184 niche). Our observations that bumblebees are less sensitive to the parasite support this hypothesis of  
185 an evolutionary adaptation. Note however that the fact that we observed a low survival of bumblebees  
186 in our experiments could be explained by the health of the colonies we received. It would be  
187 interesting to replicate these experiments on wild bumblebees although if this bias had been present, it  
188 would have influenced our results in the direction of a greater susceptibility to the parasite.

189

190 Interestingly, the effects of *M. robertsii* were the strongest on the yellow-legged hornets. Our  
191 observation that only half of the hornets survived four days after the exposition to spores is  
192 comparable to the results in Poidatz et al. (2018), thereby confirming the efficiency of *M. robertsii*  
193 against hornets. From a biocontrol perspective, the application method of *M. robertsii* seems key to  
194 enhancing selectivity: the direct application of spores into hornets' nests could be interesting as it

195 allows a selective application and the reduction of potential non-targeted species contamination.  
196 Moreover, it is possible that *M. robertsii* causes a range of early post-infection sublethal effects that  
197 impair hornet activity before they die. Indeed, many parasites and pathogens can alter the behavior of  
198 insects (Gómez-Moracho et al. 2017). *M. anisopliae* can alter the reproduction of cockroaches  
199 (*Blattella germanica*) (Quesada-Moraga et al. 2004), the nutrition of fruit flies (*Drosophila*  
200 *melanogaster*) (Toledo-Hernández et al. 2018) and also the locomotion activity of termites (Hassan et  
201 al. 2021). It is, for instance, possible that contaminated hornets are less able to leave their nest and  
202 forage, thus greatly limiting the opportunities of contaminating non-targeted species through contact  
203 with water, flowers, or honey bee hives. However, even when intraspecific contamination occurs, if  
204 the quantity of spores deposited on the surface is lower enough, it should be harmless to non-targeted  
205 species as our results demonstrate that bumblebees and wasps were not impacted by a low  
206 concentration of spores. The sublethal effects of *M. robertsii* on hornets, their ability to spread spores  
207 in the environment, and the realistic spores' transmission to other pollinators through contaminated  
208 surfaces should therefore be further taking into consideration to establish the safety of this biocontrol  
209 agent. Further studies are also necessary to assess the actual spores' quantity to inject in hornets' nests  
210 which will be dependent on the parasite transmission between nestmates. Many social insects use  
211 behavioral strategies to limit the transmission of parasites or pathogens in the colony and if so, to limit  
212 the establishment of parasites and pathogens in the nest such as grooming or reduced contact with  
213 infected individuals (Cremer et al. 2007). A preliminary experiment suggests hornets increase the  
214 duration of their grooming behavior when infected by *M. robertsii* (personal observations). These  
215 hygienic behaviors and potential others could be restrictive in an entomopathogen use context.  
216  
217 Our study suggests entomopathogenic fungi, and particularly *M. robertsii* strains, are good candidates  
218 for biocontrol of invasive yellow-legged hornets as the higher mortality found compared to the two  
219 non-target species reinforced its potential. However, as the effect on non-targeted species is  
220 concentration-dependent, there is a need to quantify the real risk of spores spreading in the  
221 environment by an infected individual to better assess the safety of this biological control agent on  
222 local biodiversity.

223

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317 **Tables**

318 **Table 1:** Details of all the insect colonies used.

	<b>Hornet</b>	<b>Wasp</b>	<b>Bumblebee</b>
<b>European evolution history</b>	Invasive	Non-invasive	Non-invasive
<b>Nest</b>	Aerial	Underground Occasionally aerial	Underground
<b>Origin</b>	Wild	Wild	Commercial
<b>Number of nests</b>	4	1	4
<b>Origin (Department code)</b>	Laburgade (46) Bouliac (33) Bouliac (33) Cahors (46)	Saucats (33)	Netherlands Netherlands Netherlands Netherlands
<b>Collection date</b>	2021-10-12 2021-10-18 2021-10-25 2021-11-28	2021-12-10	2021-06-08 2021-06-08 2021-06-08 2021-06-08
<b>Approximate nest size (cm)</b>	<30 30-80 30-80 >80	60-70	<20 <20 <20 <20
<b>Number of microcolonies</b>			
<i>Control</i>	9	6	6
$10^3$ sp/ml	9		6
$10^7$ sp/ml	9	6	6

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328 **Table 2:** Comparison of survival probability across treatment and for each species using Cox model.  
329 Treatments: C (control), L (low spore concentration), H (high spore concentration). HR (Hazard  
330 Ratio): the risk of an event occurring (in this case, the death of an individual) when comparing one  
331 treatment with another, CI (Confidence Interval): 95% confidence interval and p-value (the  
332 probability of this risk occurring at random).

<i>Species</i>	<i>Compared treatment</i>	<i>HR</i>	<i>CI</i>	<i>p-value</i>
<i>Hornet</i>	<i>C v.s. L</i>	3.16	1.21-8.24	0.0484
	<i>C v.s. H</i>	36.83	14.58-93.08	<0.001
	<i>L v.s. H</i>	11.66	5.50-24.72	<0.001
<i>Wasp</i>	<i>C v.s. H</i>	6.43	3.32-12.45	<0.001
	<i>C v.s. L</i>	1.92	0.87-4.21	0.238
<i>Bumblebee</i>	<i>C v.s. H</i>	4.01	2.23-7.21	0.014
	<i>L v.s. H</i>	1.61	0.76-3.44	0.431

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345 **Table 3:** Comparison of survival probability across species and for each treatment using Cox models.  
346 Treatments: C (control), L (low spore concentration), H (high spore concentration). HR (Hazard  
347 Ratio): the risk of an event occurring (in this case, the death of an individual) when comparing one  
348 species with another, CI (Confidence Interval): 95% confidence interval and p-value (the probability  
349 of this risk occurring at random).

<i>Treatment</i>	<i>Compared species</i>	<i>HR</i>	<i>CI</i>	<i>p-value</i>
<i>Control</i>	<i>H v.s. W</i>	1.38	0.23-8.46	0.935
	<i>H v.s. B</i>	10.62	2.07-54.44	0.0128
	<i>W v.s. B</i>	7.69	1.28-46.26	0.0666
<i>Low concentration</i>	<i>H v.s. B</i>	8.35	3.08-22.67	<0.001
	<i>H v.s. W</i>	0.44	0.25-0.72	0.004
<i>High concentration</i>	<i>H v.s. B</i>	1.75	1.05-2.92	0.083
	<i>W v.s. B</i>	4.13	2.31-7.40	<0.001

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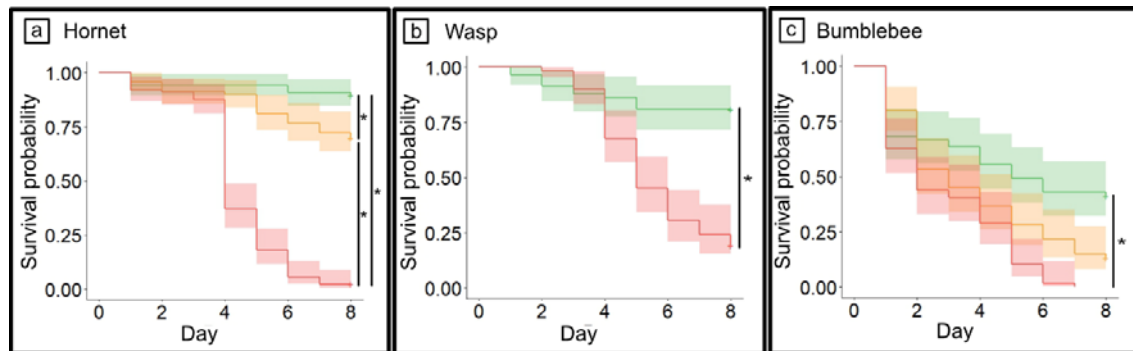
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367 **Figure**

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371 **Fig. 1:** Survival probability of the three Hymenoptera tested during eight days: a) hornet, b) wasp, c)  
372 bumblebee. We exposed the insects to three treatments: control (green and upper line), low  
373 concentration of spores ( $10^3$  sp/ml, orange and middle line when three lines are present), high  
374 concentration of spores ( $10^7$  sp/ml, red and lower line). Solid lines represent experimental data and  
375 colored areas, the 95% confidence interval. Stars represent significant differences between groups  
376 calculated with a mixed-effect Cox model (a,c) or a Cox proportional hazards regression model (b).

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