

1 Synergistic effects of the insecticide flupyradifurone and the entomopathogen 2 *Metarhizium brunneum* in ants

3
4 Daniel Schläppi^{1*}, Adam Al-Hashemi¹, Vaneeza Wasif¹, Florent Masson¹, Nathalie Stroeymeyt¹

5
6 ¹ School of Biological Sciences, University of Bristol, Life Sciences Building, 24 Tyndall Avenue, BS8 1TQ Bristol, UK

7
8 * Corresponding Author: d.schlaepi@mail.ch

9 10 Abstract

11 Agricultural pesticides such as commonly used neonicotinoids are major drivers of the global entomofauna
12 declines. Furthermore, synergistic interactions between pesticides and other stressors substantially amplify
13 their environmental effects and could therefore have important implications for policy decisions that aim to
14 improve the health of insects. Due to increasing restrictions for neonicotinoid use in the EU and rising pest
15 resistance, alternatives were introduced, such as flupyradifurone, a novel systemic butenolide insecticide that
16 was initially promoted as safe for non-target insects. However, this is increasingly questioned by the
17 accumulation of evidence for harmful effects on pollinators. Furthermore, studies investigating effects in other
18 non-target arthropods are lacking. We therefore investigated how chronic exposure to flupyradifurone affects
19 survival of black garden ants *Lasius niger*. Furthermore, we studied potential interactive effects of field realistic
20 flupyradifurone doses with exposure to the entomopathogenic fungus *Metarhizium brunneum*. We found that
21 concentrations exceeding 50 ppm result in increased worker mortality, and that field realistic contamination
22 did not reduce food intake. At low concentrations, flupyradifurone exposure alone did not affect survival, but
23 chronic flupyradifurone exposure at sublethal doses increased pathogen susceptibility in ants, indicating a
24 synergistic effect of the two stressors. This first report of synergistic effects between flupyradifurone and
25 pathogens raises further concerns about the long-term impacts of novel pesticides on the health of beneficial
26 insects.

27 **Keywords:** chronic exposure, field realistic, pesticide, stressor interaction, sublethal effects

29 **Introduction**

30 Global insect biodiversity and abundance are being lost at alarming rates (Cardoso et al., 2020; Sánchez-
31 Bayo & Wyckhuys, 2019; Wagner, 2020). A major driver of these declines is pollution with agrochemicals
32 such as neonicotinoid insecticides (Goulson et al., 2015; Raven & Wagner, 2021; Sánchez-Bayo et al., 2016;
33 Sánchez-Bayo & Wyckhuys, 2019). Insects are important biological indicators for ecosystem health and they
34 provide vital ecosystem services such as pollination, seed dispersal, pest control, and nutrient cycling (Elizalde
35 et al., 2020; Losey & Vaughan, 2006).

36 Whilst adverse impacts of neonicotinoids on beneficial insects have been long known (Desneux et al.,
37 2007; Pisa et al., 2015; Stanley & Preetha, 2016), environmental risk assessments have typically been based on
38 acute toxicity assays and often failed to detect sublethal effects. Reported effects of sublethal exposure include
39 detrimental impacts on foraging and other behaviours, cognitive abilities, immune functions, colony
40 development, fertility, and social interactions (Blacquiere et al., 2012; Pisa et al., 2015; Schläppi, Stroeymeyt,
41 et al., 2021; Wood & Goulson, 2017). Mounting evidence for negative impacts of neonicotinoids on pollinators
42 coupled with increasing public awareness and media attention led to a restriction of the use of clothianidin,
43 thiamethoxam, and imidacloprid in the European Union in 2013 (Epstein et al., 2021). To compensate for the
44 neonicotinoid ban, the next generation of pesticides was introduced to the market, including flupyradifurone
45 (FPF), a butenolide insecticide which was approved for the European Union in 2015 (Nauen et al., 2015).
46 Although structurally different to neonicotinoids, it is also an agonists of insect nicotinic acetylcholine
47 receptors (nAChRs) modulating ion channels expressed in the central nervous system of insects, thereby
48 making it highly effective against a broad range of pests (Nauen et al., 2015; Tomizawa & Casida, 2005). Given
49 this is the same mode of action as its predecessors, questions arise about adverse effects on non-target insects.
50 Despite its approval for use in the US and Europe and the initial claim for it to be “bee safe” due to its
51 ecotoxicological profile, recent evidence suggest that flupyradifurone is a risk for pollinators (Siviter & Muth,
52 2020; Tosi et al., 2021). However, the literature focuses primarily on bees while research on other non-target
53 insects is lacking. Ants, for example, are exposed to insecticides in a similar fashion to bees and provide
54 essential ecosystem services important for food security (Del Toro et al., 2012; Gras et al., 2016; Hölldobler &
55 Wilson, 1990), which confers them immense economic and ecological value.

56 Another major concern of neonicotinoid insecticides are interactions with other biological stressors as
57 synergistic interaction between multiple stressors may amplify their environmental significance (Folt et al.,
58 1999; Siviter et al., 2021). Exposure to multiple stressors such as parasite pressure, malnutrition, or climate
59 change, is likely to happen frequently in natural conditions and, consequently, their interactive effects need to
60 be taken into account to assess the impact of pesticides on non-target insects accurately (Annoscia et al., 2020;
61 Kaunisto et al., 2016; Tosi et al., 2017). Some pesticides have been shown to act as immune suppressors,
62 thereby enhancing the spread and deleterious effects of pathogens in bees (Doublet et al., 2015; Sánchez-Bayo
63 et al., 2016; T O’Neal et al., 2018). The neonicotinoids thiamethoxam and its metabolite clothianidin for
64 example affect honey bee immunocompetence and enhance their susceptibility to viruses (Coulon et al., 2019;
65 Di Prisco et al., 2013). By contrast, little is known on the interactions of FPF with other stressors. It is therefore

66 the aim of this study to investigate the effects of chronic FPF exposure on ants and explore potential interactive
67 effects with pathogens.

68 To investigate adverse effects of FPF exposure alone or in combination with another stressor we devised
69 two experiments using black garden ants *Lasius niger* as model species. First, we exposed workers to a range
70 of FPF concentrations to determine the susceptibility of *L. niger* and establish a range of sublethal doses, and
71 tested whether ants exhibited preferences for, were neutral to, or avoided sugary solutions contaminated with
72 FPF. Second, we conducted a fully crossed experiment with sublethal flupyradifurone exposure and challenge
73 with a fungal pathogen to investigate potential interactive effects between the two stressors.

74 **Methods**

75 *L. niger* colonies were initiated using newly mated queens collected in Berlin in July 2021. The colonies
76 were raised at 25°C and 65% humidity, with a 12h day/night cycle, *ad libitum* supply of water and honey water
77 (15% mass fraction of honey) and weekly provision of *Drosophila hydei* fruit flies.

78 *Flupyradifurone susceptibility test*

79 Four stock colonies of around 150 workers were selected and from each 120 individuals were sampled
80 and assigned to eight subsets of fifteen workers in a stratified random way (n=480 ants across 32 subsets). Each
81 subset was kept in a separate petri dish ($\varnothing = 50$ mm) sealed with parafilm and with fluon-coated walls to
82 prevent the ants from escaping. The floor of each petri dish was covered by a thin layer of plaster of Paris
83 soaked with 1 mL of water to provide humidity. After one day of acclimatisation, the eight subsets from each
84 colony were randomly allocated to the control treatment or one of seven FPF treatments (see below). Upon
85 initiation of the experiment and then every 3 days each subset received a freshly prepared feeding solution
86 provided in a 200 μ L tube wrapped in aluminium foil and mounted on a plastic tray (20x10 mm) with Blu Tack.
87 Cotton balls were used to close the feeding tubes to prevent leaking or drowning. Survival was then checked
88 daily for two weeks by counting and removing dead ants; the experimenters were blind to treatment during all
89 survival checks.

90 In the field, ants will be exposed to pesticides via a combination of contact with spray droplets,
91 contaminated soils, foliage, and water as well as oral uptake of contaminated water and foods such as nectar,
92 pollen, guttation fluid and seeds, honeydew secreted by aphids and some scale insects, and preys that were
93 directly or indirectly exposed (Schläppi, Stroeymeyt, et al., 2021). True field-realistic exposure of ants has yet
94 to be determined. However, given that data of the Environmental Protection Agency (EPA) showed residues
95 up to 36 ppm in flowers and up to 4.3 ppm in nectar, and that combined exposure via multiple exposure routes
96 adds up, we considered 5 ppm to be a conservative field-realistic exposure dose and designed our concentration
97 range around that. Thus, fresh treatment feeding solutions were prepared on each feeding day by mixing a 30%
98 honey water solution with a 1000ppm FPF stock solution in MiliQ water to produce 8 different solutions with
99 final concentrations of 15% honey water each and 0 (control), 0.5, 1, 5, 10, 50, 100 or 500 ppm Flupyradifurone
100 (Sigma-Aldrich PESTANAL® analytical standard, 99.5% purity).

101 *Meal size quantification*

102 To determine whether FPF affects honey water uptake, we fed ants with 15% honey water at four different
103 FPF concentrations. The food uptake assay was adapted from a protocol for quantifying mosquito meals (Jové
104 et al., 2020): a fluorescein dye (see below) was added to the feeding solution to allow for subsequent meal size
105 quantification by measuring fluorescence levels in crushed ants.

106 Workers were sampled from 5 source colonies, each donating 110 individuals. Ants from each colony
107 were then split into four groups of equal size and distributed to petri dishes in a stratified random way. The
108 groups (N=20) were then pseudo-randomly assigned to 4 treatments and kept in the incubator for three days
109 without food. Subsequently, a tube of treatment solution was provided as described above, but a fraction of
110 water got replaced by a fluorescein sodium salt (Sigma-Aldrich) stock solution at 2% in MiliQ water to create
111 four solutions with final concentrations of 15% honey water 0.01% fluorescein and 0, 5, 50 or 500 ppm
112 flupyradifurone. The workers had access to the feeding solution for 24h before being frozen at -80°C for 30
113 minutes. Subsequently, ants were homogenized individually in racked collection tubes (Qiagen) containing 100
114 µL of phosphate-buffered saline (PBS) buffer and a 2 mm glass bead each. Crushing was performed using a
115 tissue lyser at 30 Hz for two runs of 1 minute with rotation of the plate between the two runs. Following 2
116 minutes of centrifugation at 3000 rpm 20 µL of the supernatant and 80 µL PBS buffer were transferred into 96-
117 well plates. Fluorescence was measured on a SpectraMax iD5 plate reader with excitation at 485 nm and
118 emission at 535 nm. Each measure was averaged from 4 reading points 1.5 mm apart, with 400 ms integration
119 time and a gain of 500 volt.

120 Quantification was done using a standard curve, which was obtained using an aliquot of the control
121 treatment feeding solution containing 0.01% fluorescein and 0 FPF, which was treated identically to the meals
122 that were served to the ants (same light and temperature conditions throughout the duration of the experiment,
123 and subsequent freezing). Reference standard curves were prepared from the retained aliquot by making a serial
124 dilution for 8 solutions containing 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, or 0 µL of 0.01% fluorescein
125 treatment solution mixed with PBS to a final total volume of 100 µL. An unfed ant treated identically to the
126 samples was used as a blank. To calculate the meal volumes, mean fluorescence value of blanks was subtracted
127 from all other samples and consumed volume was extrapolated from the standard curves.

128 *Flupyradifurone and fungus co-exposure*

129 To test for potential interactive effects between FPF and fungal pathogens, ants were chronically exposed
130 to sublethal flupyradifurone doses for ten days and then subsequently exposed to a lethal dose of fungal spores.
131 We used the entomopathogenic fungus *Metarhizium brunneum* that is common in soil environments and
132 naturally infects *L. niger*, which the ants combat with various physiological and behavioural defences (Cremer,
133 2019; Schluns & Crozier, 2009; Stroeymeyt et al., 2018). A preliminary test was conducted to confirm that
134 FPF does not directly interfere with fungal germination (supplementary fig 1).

135 Workers from six stock colonies were evenly distributed into 15 petri dishes as described above
136 (44 workers per dish, 660 workers total). Petri dishes were pseudo-randomly assigned to one of three treatment
137 groups (0, 5 and 50 ppm of FPF, that is, control, low sublethal dose, and high sublethal dose, respectively).

138 Feeding solutions were freshly prepared and provided every 3 days as described above. After ten days, workers
139 of each petri dish were randomly selected and split into two groups of twenty, one assigned to a fungus exposure
140 and the other to the sham treatment, resulting in a total of six treatment groups. Fungal exposures were
141 performed using standard procedures for the generalist entomopathogen *M. brunneum*, strain MA275, KVL
142 03-143 (Stroeymeyt et al., 2018). *M. brunneum* was cultured on Sabouraud-Dextrose Agar (SDA) plates at
143 24°C for 3 weeks or until sporulation was observed. Conidiospores were harvested in 0.05% Triton-X100,
144 washed twice and adjusted to 10^9 spores/mL in 0.05% Triton-X100 to create a spore stock suspension. For the
145 exposure, the stock suspension was diluted by half with 0.05% Triton-X100 resulting in a final spore
146 concentration of 5×10^8 . Individuals were placed on ice to reduce their movement and then 0.3 μ L of the spore
147 solution was topically pipetted onto their gaster. Sham treated ants were exposed with the same volume of
148 0.05% Triton X-100 only. They were left to dry for 3 minutes and then transferred individually into new petri
149 dishes. The order of exposure was organized in a stratified random way to minimize time and cohort effects
150 between treatment groups, as well as being split over 2 blocks on consecutive days. Petri dishes ($\varnothing = 35$ mm,
151 N=600) were prepared in advance with fluon coating and a water tube (200 μ L Eppendorf tubes fastened with
152 Blu Tack and closed with a cotton ball). These petri dishes were then sealed with parafilm and placed in the
153 incubator. Survival was then checked daily for two weeks by counting and removing dead ants; the
154 experimenters were blind to treatment during all survival checks.

155 *Statistical Analyses*

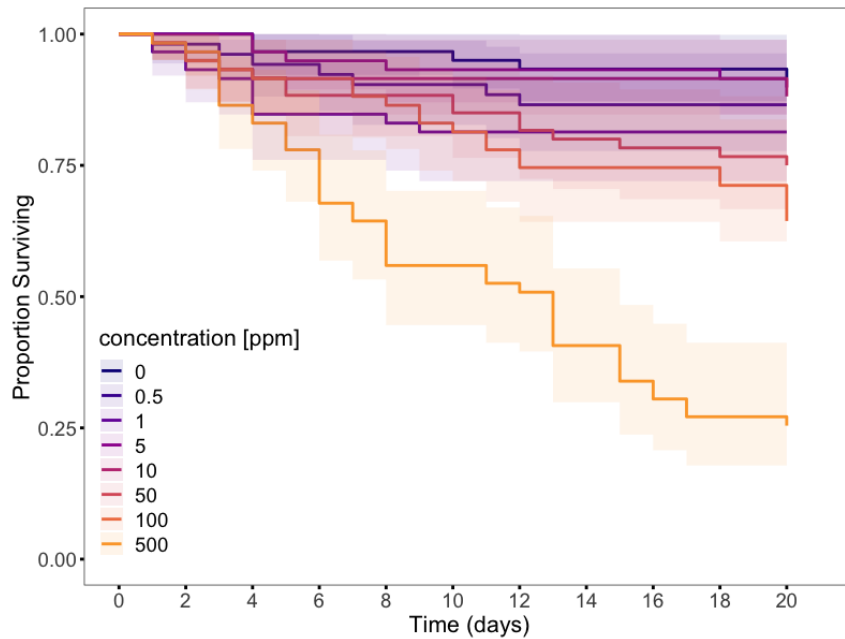
156 All statistical analyses were performed in using R v4.2.2 (R Core Team, 2020). Survival analyses were
157 performed using Cox proportional hazards mixed-effect models using R packages survival (Thernau, 2022a)
158 and coxme (Thernau, 2022b). For the meal size quantification post-hoc comparisons between treatments were
159 performed using R package mutlcomp (Hothorn et al., 2008), and p-values were corrected for multiple
160 comparisons using the Benjamini-Hochberg method. For the FPF susceptibility test concentration was used as
161 fixed effect and petri dish and stock colony as random effects. For the interaction experiment, FPF
162 concentration, fungus treatment and their one-way interaction were used as fixed effects and petri dish, stock
163 colony and exposure block as random effects.

164 A generalized linear mixed model (GLMM) with a negative binomial distribution was used to test for
165 differences in meal size depending on FPF concentration (R package lme4). Treatment was included as fixed
166 effect and petri dishes, colonies, as well as block were included as random.

167 **Results**

168 *Susceptibility test*

169 Flupyradifurone concentration had a significant effect on ant mortality (Fig. 1; Cox proportional hazards
170 mixed effect model, effect of FPF concentration: coef = 0.0043, exp(coef) = 1.0043, z = 6.21, p < 0.001).



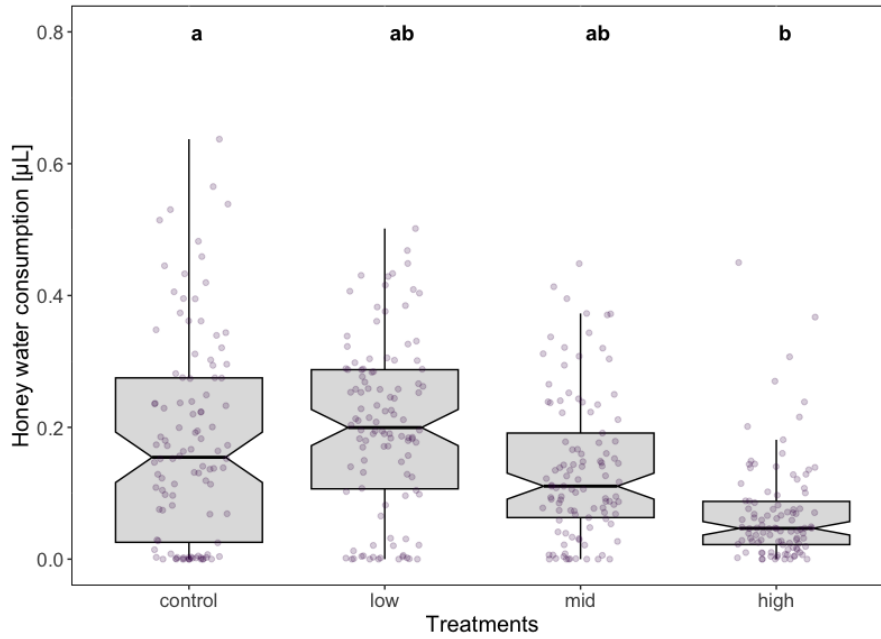
171 **Figure 1: Flupyradifurone susceptibility.** Lines represent the proportion of *L. niger* workers surviving as a function
172 of time during chronic exposure to different concentrations of FPF (total sample size: N=467). Shaded areas represent
173 the 95% confidence interval around each line. Concentration had a significant effect on survival (Cox proportional hazards
174 model; $\chi^2 = 31.699$, $df = 7$, $p < 0.001$).

175 *Meal size quantification*

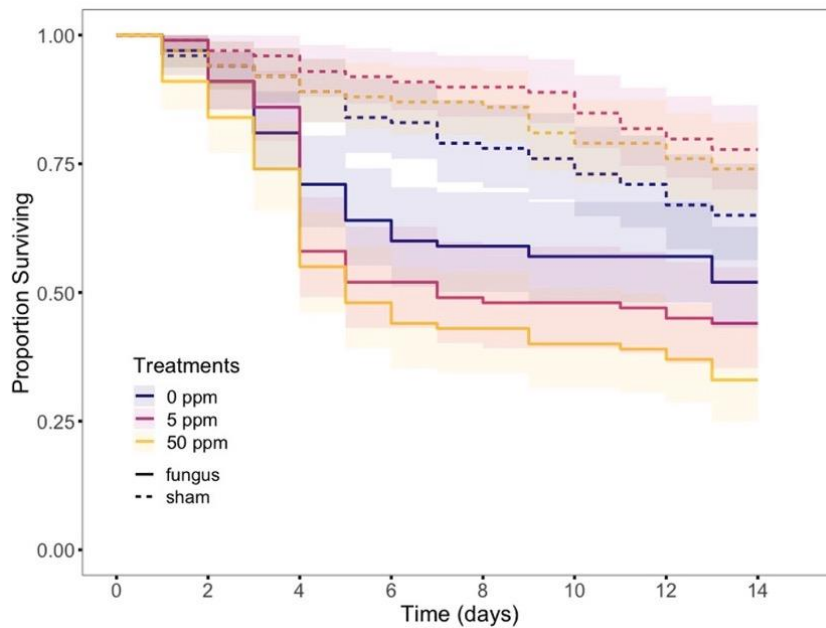
176 GLMM analysis revealed that meal size, i.e. consumed volume of honey water, was significantly different
177 among treatments ($\chi^2 = 7.853$, $df = 3$, $p = 0.049$). Pairwise comparisons using Tukey adjustment indicated that
178 ants in the treatment with the highest FPF concentration (500 ppm) consumed lower volumes ($0.07 \pm 0.08 \mu\text{L}$)
179 compared to the controls ($0.179 \pm 0.16 \mu\text{L}$; $p < 0.05$; Fig. 2). The remaining pairwise comparisons with the
180 low and the mid treatment ($0.2 \pm 0.13 \mu\text{L}$ and $0.14 \pm 0.11 \mu\text{L}$ respectively) were not significant.

181 *Flupyradifurone and fungus co-exposure*

182 As expected, whilst chronic exposure to sublethal FPF concentration did not affect ant survival (coef = -
183 0.0027, $\exp(\text{coef}) = 0.9973$, $z = -0.54$, $p = 0.59$), exposure to a lethal dose of *M. brunneum* fungal spores
184 significantly increased mortality (coef = 0.8271, $\exp(\text{coef}) = 2.2867$, $z = 4.76$, $p < 0.001$). Furthermore, there
185 was a marginally significant interaction between FPF concentration and fungal exposure (coef = 0.0117,
186 $\exp(\text{coef}) = 1.0118$, $z = 1.95$, $p = 0.051$), indicating that exposure to increasing sublethal doses of FPF
187 significantly increased mortality following pathogen challenge (Fig. 3).



188 **Figure 2: Meal size.** Volume (μL) of sugar water consumed by *Lasius niger* workers in 24h at different
189 concentrations of FPF (control = 0, low = 5, mid = 50, high = 500 ppm). Boxplots are shown with the inter-quartile-ranges
190 (box), medians (black line in box) and outliers (dots). Transparent dots represent individual data points and bold letters
191 (a,b) indicate significant differences ($p < 0.05$) between treatments (Tukey post hoc test).



192 **Figure 3: Interactive effects between Flupyradifurone and *M. brunneum*.** Lines represent the proportion of *L.*
193 *niger* workers surviving as a function of time following exposure to live *M. brunneum* spores (solid lines) or to a sham
194 solution (dashed lines) after ten days chronic exposure to different concentrations of flupyradifurone (0, 5, and 50 ppm).
195 Time indicated is post exposure to the fungus or sham treatment. Shaded areas represent the 95% confidence interval
196 around each curve.

197 Discussion

198 This study assessed the effect of FPF on black garden ants (*L. niger*) by quantifying mortality under
199 chronic exposure showing increased mortality with increasing FPF concentration. Additionally, we found that
200 ants were not repelled by sublethal doses of FPF, which indicates that they can effectively ingest FPF present
201 in their environment. Furthermore, we showed that a challenge with *M. brunneum* was more likely to be lethal
202 for ants that were previously exposed to the insecticide, indicating a synergistic effect of the two stressors.

203 FPF concentrations exceeding 50 ppm resulted in significantly increased worker mortality in the
204 susceptibility test, while concentrations of 50 ppm or lower showed comparable mortality rates to the control
205 group and are henceforth considered sublethal doses according to the definition in (Desneux et al., 2007). For
206 bees the oral LD₅₀ is specified to be 1-1.2 µg ai/bee ('active ingredient'; Baltiansky et al., 2021; Nauen et al.,
207 2015; Gladbach et al., 2013). At 100 ppm with a feeding volume of 0.16 µL (estimated from our meal size
208 quantification), ants would ingest around 16 ng in 24h. It is generally assumed that body mass and xenobiotics
209 susceptibility are correlated, meaning that arthropods respond to insecticides in direct proportion to their body
210 mass (Robertson et al., 2017). With this assumption and the approximation that black garden ants weigh
211 roughly 100 times less than honey bee workers (Schläppi et al., 2020; Zóltowska et al., 2011), our finding
212 suggest that FPF susceptibility in ants is in the same order of magnitude as in bees.

213 It is worth noting that the true level of exposure of ants to such concentrations in their environment remains
214 uncertain. Residues in pollen and nectar are considered to be the main exposure routes for bees and their
215 reported residue values are as high as 68 ppm for pollen and 21.8 ppm for extrafloral nectaries (Glaberman &
216 White, 2014). Many studies with honey bees use 4 ppm to test for effects of field-realistic doses as nectar
217 collected by returning foragers contained 4.3 ppm of FPF (e.g. (Siviter & Muth, 2022; Wu et al., 2021). Our
218 sublethal concentrations for ants (0.5 - 50 ppm) fall within this range, suggesting that FPF residue values in the
219 field may not increase ant mortality via direct toxicity. However, future studies should quantify true field-
220 realistic exposure for ants, based on a combination of all relevant exposure routes. Furthermore, season-long
221 exposure might aggravate the effect of FPF (Long & Krupke, 2016), as prolonged exposure can increase
222 toxicity and subtle sublethal effects may add up (Sánchez-Bayo & Tennekes, 2020). For example, chronic
223 exposure of *L. niger* colonies to the neonicotinoid thiamethoxam results in colony-wide effects that become
224 apparent only in the second year of colony development (Schläppi et al., 2020). Similarly, in honey bees, long-
225 term monitoring facilitated the detection of lethal and sublethal effects of chronic flupyradifurone exposure at
226 doses 100 folds lower than oral LD₅₀ (Tosi et al., 2021). Consequently, long-term studies with entire colonies
227 are of utmost importance and unavoidable to understand pesticide impacts on social insects.

228 In contrast to honey bees, which respond to field-realistic concentrations of FPF by a decreased food intake
229 and erratic foraging behaviour (Hesselbach et al., 2020; Wu et al., 2021), we found that ants reduce their food
230 uptake only at the highest concentration of FPF (500 ppm). Our results suggest that ants are not repelled by
231 field-realistic concentrations of FPF. However, the absence of a repellent effect implies that the ants are prone
232 to sharing contaminated food among nestmates, with potentially severe consequences for the colony. The
233 insecticide is thus likely to reach all members of the colony, including the queen. Even though the queen might

234 have some protection via the colony or potentially superior detoxification compared to workers (Schläppi et
235 al., 2020), she will still get exposed repeatedly over extended periods and thus might face a trade-off between
236 detoxification and reproduction (Schwenke et al., 2016).

237 Our study contributes to the understanding of stressor interactions by demonstrating synergistic effects
238 between FPF and a pathogen for the first time. At sublethal concentrations, FPF exposure alone did not affect
239 survival, but it increased mortality when combined with *M. brunneum*. This suggests that chronic exposure to
240 sublethal doses of FPF can increase pathogen susceptibility in ants. Our findings align with previous findings
241 about immune-suppressing effects of other pesticides that enhance the spread and deleterious effects of
242 pathogens in bees (Coulon et al., 2019; Di Prisco et al., 2013; Doublet et al., 2015; Sánchez-Bayo et al., 2016;
243 T O’Neal et al., 2018). As we did not investigate immune functions it is not possible to identify the underlying
244 mechanism for the synergistic effect. FPF could either cause a direct immune suppression, or it might trigger a
245 resource allocation trade-off between immune functions and detoxification (Collison et al., 2016). As agonist
246 of nAChRs, FPF disrupts the function of the central nervous system and could also alter physiological and
247 behavioural strategies that protect colonies against diseases (Cremer et al., 2018). Ants benefit from social
248 immunity, a suite of collective disease defence behaviours (Cremer et al., 2018), and superorganism resilience
249 (Straub et al., 2015). Effects found for individuals do not necessarily translate to the colony level (Schläppi,
250 Kettler, et al., 2021). Consequently, future studies should investigate whether FPF exposure increases pathogen
251 susceptibility even more at colony level because it impairs colony-wide immunity protection, or conversely,
252 the same protections could effectively reduce the effect of FPF.

253 This study highlights the ecotoxicological risk imposed by insecticides. Our findings are particularly
254 concerning as they suggest that FPF, despite its initial perception of relative safety for bees, poses a comparable
255 threat to beneficial insects as banned neonicotinoids. Our findings highlight the possibility of divergent
256 responses between ants and bees, despite their similarities, emphasizing the need to account for their distinct
257 behaviours and ecological traits. Consequently, ants should be included as representative model organisms in
258 environmental risk assessments for agrochemicals alongside bees. Furthermore, the results emphasize once
259 more the significance of considering the interactive effects of multiple stressors when evaluating the risks of
260 agrochemicals. The unfolding narrative for “next generation” insecticides closely resembles the patterns
261 observed for banned neonicotinoids. Sulfoxaflor, introduced to the market alongside FPF, has already faced
262 prohibition due to similar concerns and our results indicate that FPF might be just as concerning. This underlines
263 the necessity of rigorous evaluation of novel pesticides *prior* to their release to safeguard insect biodiversity
264 and preserve ecosystem functioning.

265 **Data availability**

266 Data supporting the findings of this study will be published on FigShare upon publication of the
267 manuscript. Until then, they are available from the corresponding author upon reasonable request.

268 **Acknowledgements**

269 Financial support was provided to DS by the Swiss National Science Foundation (P2BEP3_195575 &
270 P500PB_206883) and to N.S. by the European Research Council (ERC Starting Grant ‘DISEASE’, no.
271 802628).

272 **Author Contributions**

273 D.S. conceived the study and designed it with N.S. and F.M; D.S., V.W., and A. AH. conducted the
274 research and performed the experiments with inputs from N.S. & F.M.; N.S. provided laboratory space and
275 materials; D.S., V.W., and A. AH. analysed the data with inputs from N.S.; D.S. wrote the manuscript based
276 on a first draft by A. AH, with contributions from all authors. All authors edited and approved the manuscript.

277 **Competing Interests statement**

278 The authors declare no conflict of interest.

279

280 References

- 281 Annoscia, D., Di Prisco, G., Becchimanzi, A., Caprio, E., Frizzera, D., Linguadoca, A., . . . Pennacchio, F. (2020).
282 Neonicotinoid Clothianidin reduces honey bee immune response and contributes to *Varroa* mite
283 proliferation. *Nature Communications*, *11*(1), 1-7. <https://doi.org/10.1038/s41467-020-19715-8>
284 Baltiansky, L., Sarafian-Tamam, E., Greenwald, E., & Feinerman, O. (2021). Dual fluorescence imaging and
285 automated trophallaxis detection for studying multinutrient regulation in superorganisms. *Methods in*
286 *Ecology and Evolution*. <https://doi.org/10.1111/2041-210X.13646>
287 Blacquiere, T., Smagghe, G., Van Gestel, C. A., & Mommaerts, V. (2012). Neonicotinoids in bees: a review on
288 concentrations, side-effects and risk assessment. *Ecotoxicology*, *21*(4), 973-992.
289 <https://doi.org/10.1007/s10646-012-0890-7>
290 Cardoso, P., Barton, P. S., Birkhofer, K., Chichorro, F., Deacon, C., Fartmann, T., . . . Hallmann, C. A. (2020).
291 Scientists' warning to humanity on insect extinctions. *Biological Conservation*, *242*, 108426.
292 <https://doi.org/10.1016/j.biocon.2020.108426>
293 Collison, E., Hird, H., Cresswell, J., & Tyler, C. (2016). Interactive effects of pesticide exposure and pathogen
294 infection on bee health—a critical analysis. *Biological Reviews*, *91*(4), 1006-1019.
295 Coulon, M., Schurr, F., Martel, A.-C., Cougoule, N., Begaud, A., Mangoni, P., . . . Ribiere-Chabert, M. (2019).
296 Influence of chronic exposure to thiamethoxam and chronic bee paralysis virus on winter honey bees.
297 *PLoS ONE*, *14*(8), e0220703. <https://doi.org/10.1371/journal.pone.0220703>
298 Cremer, S. (2019). Social immunity in insects. *Current Biology*, *29*(11), R458-R463.
299 <https://doi.org/10.1016/j.cub.2019.03.035>
300 Cremer, S., Pull, C. D., & Fuerst, M. A. (2018). Social immunity: emergence and evolution of colony-level disease
301 protection. *Annual Review of Entomology*, *63*, 105-123. [https://doi.org/10.1146/annurev-ento-020117-](https://doi.org/10.1146/annurev-ento-020117-043110)
302 [043110](https://doi.org/10.1146/annurev-ento-020117-043110)
303 Del Toro, I., Ribbons, R. R., & Pelini, S. L. (2012). The little things that run the world revisited: a review of ant-
304 mediated ecosystem services and disservices (Hymenoptera: Formicidae). *Myrmecological News*, *17*, 133-
305 146.
306 Desneux, N., Decourtye, A., & Delpuech, J.-M. (2007). The sublethal effects of pesticides on beneficial arthropods.
307 *Annual Review of Entomology*, *52*, 81-106. <https://doi.org/10.1146/annurev.ento.52.110405.091440>
308 Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., . . . Pennacchio, F. (2013).
309 Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen
310 in honey bees. *Proceedings of the National Academy of Sciences*, *110*(46), 18466-18471.
311 <https://doi.org/10.1073/pnas.1314923110>
312 Doublet, V., Labarussias, M., de Miranda, J. R., Moritz, R. F., & Paxton, R. J. (2015). Bees under stress: sublethal
313 doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle.
314 *Environmental Microbiology*, *17*(4), 969-983. <https://doi.org/10.1111/1462-2920.12426>
315 Elizalde, L., Arbetman, M., Arnan, X., Eggleton, P., Leal, I. R., Lescano, M. N., . . . Pirk, G. I. (2020). The ecosystem
316 services provided by social insects: traits, management tools and knowledge gaps. *Biological Reviews*,
317 *95*(5), 1418-1441. <https://doi.org/10.1111/brv.12616>
318 Epstein, Y., Chapron, G., & Verheggen, F. (2021). EU Court to rule on banned pesticide use. *Science*, *373*(6552),
319 290. <https://doi.org/DOI:10.1126/science.abj9226>
320 Folt, C., Chen, C., Moore, M., & Burnaford, J. (1999). Synergism and antagonism among multiple stressors.
321 *Limnology and Oceanography*, *44*(3part2), 864-877. https://doi.org/10.4319/lo.1999.44.3_part_2.0864
322 Gladbach, D., Theis, M., Przygoda, D. & Nikolakis, A. (2013). *Assessment of chronic effects of BYI02960 tech. to*
323 *the honey bee, Apis mellifera L., in a 10 days continuous laboratory feeding test; Bayer CropScience AG,*
324 *Monheim, Germany; Report No E318 4561-8, Document No: M-462475-01-1.*
325 Glaberman, S., & White, K. (2014). Environmental Fate and Ecological Risk Assessment for Foliar, Soil Drench,
326 and Seed Treatment Uses of the New Insecticide Flupyradifurone (BYI 02960). *U.S. Environmental*
327 *Protection Agency (EPA), Office of Pesticide Programs, Environmental Fate and Effects Division EFED*
328 Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from
329 parasites, pesticides, and lack of flowers. *Science*, *347*(6229), 1255957.
330 <https://doi.org/10.1126/science.1255957>
331 Gras, P., Tschardtke, T., Maas, B., Tjoa, A., Hafsa, A., & Clough, Y. (2016). How ants, birds and bats affect crop
332 yield along shade gradients in tropical cacao agroforestry. *Journal of Applied Ecology*, *53*(3), 953-963.
333 <https://doi.org/10.1111/1365-2664.12625>
334 Hesselbach, H., Seeger, J., Schilcher, F., Ankenbrand, M., & Scheiner, R. (2020). Chronic exposure to the pesticide
335 flupyradifurone can lead to premature onset of foraging in honeybees *Apis mellifera*. *Journal of Applied*
336 *Ecology*, *57*(3), 609-618. <https://doi.org/10.1111/1365-2664.13555>
337 Hölldobler, B., & Wilson, E. O. (1990). *The ants*. Springer. <https://doi.org/10.1046/j.1420-9101.1992.5010169.x>

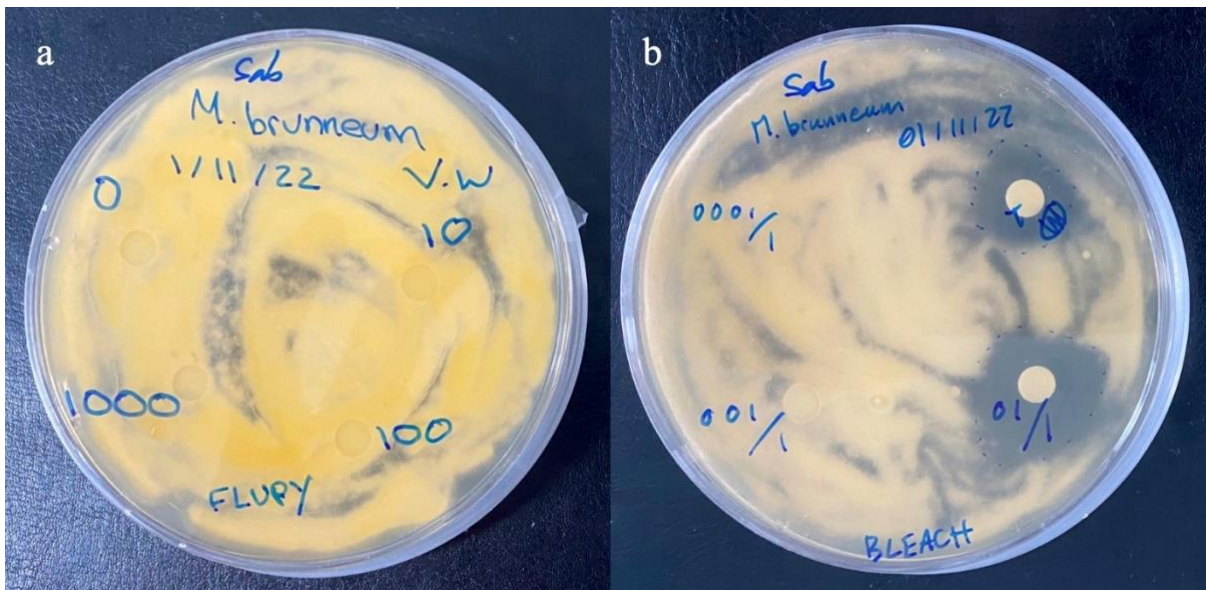
- 338 Hothorn, T., Bretz, F., Westfall, P. (2008). Simultaneous Inference in General Parametric Models. *Biometrical*
339 *Journal*, 50(3), 346-363.
- 340 Jové, V., Venkataraman, K., Gabel, T. M., & Duvall, L. B. (2020). Feeding and quantifying animal-derived blood
341 and artificial meals in *Aedes aegypti* mosquitoes. *Journal of Visualized Experiments: JoVE*(164).
342 <https://doi.org/10.3791/61835>
- 343 Kaunisto, S., Ferguson, L. V., & Sinclair, B. J. (2016). Can we predict the effects of multiple stressors on insects in
344 a changing climate? *Current Opinion in Insect Science*, 17, 55-61.
345 <https://doi.org/10.1016/j.cois.2016.07.001>
- 346 Long, E. Y., & Krupke, C. H. (2016). Non-cultivated plants present a season-long route of pesticide exposure for
347 honey bees. *Nature Communications*, 7(1), 11629. <https://doi.org/10.1038/ncomms11629>
- 348 Losey, J. E., & Vaughan, M. (2006). The Economic Value of Ecological Services Provided by Insects. *Bioscience*,
349 56(4), 311-323. [https://doi.org/10.1641/0006-3568\(2006\)56\[311:TEVOES\]2.0.CO;2](https://doi.org/10.1641/0006-3568(2006)56[311:TEVOES]2.0.CO;2)
- 350 Nauen, R., Jeschke, P., Velten, R., Beck, M. E., Ebbinghaus-Kintscher, U., Thielert, W., . . . Raupach, G. (2015).
351 Flupyradifurone: a brief profile of a new butenolide insecticide. *Pest Management Science*, 71(6), 850-862.
352 <https://doi.org/10.1002/ps.3932>
- 353 Pisa, L. W., Amaral-Rogers, V., Belzunces, L. P., Bonmatin, J.-M., Downs, C. A., Goulson, D., . . . McField, M.
354 (2015). Effects of neonicotinoids and fipronil on non-target invertebrates. *Environmental Science and*
355 *Pollution Research*, 22(1), 68-102. <https://doi.org/10.1007/s11356-014-3471-x>
- 356 R Development Core Team (2020). R: A language and environment for statistical computing. R Version 4.2.2. R
357 Foundation for Statistical Computing. (Vienna, 2018). <http://cran.r-project.org>.
- 358 Raven, P. H., & Wagner, D. L. (2021). Agricultural intensification and climate change are rapidly decreasing insect
359 biodiversity. *Proceedings of the National Academy of Sciences*, 118(2), e2002548117.
360 <https://doi.org/10.1073/pnas.2002548117>
- 361 Robertson, J. L., Jones, M. M., Olguin, E., & Alberts, B. (2017). *Bioassays with arthropods*. CRC press.
- 362 Schluns, H., & Crozier, R. H. (2009). Molecular and chemical immune defenses in ants (Hymenoptera: Formicidae).
363 *Myrmecological News*, 12, 237-249.
- 364 Schläppi, D., Kettler, N., Glauser, G., Straub, L., Yañez, O., & Neumann, P. (2021). Varying impact of neonicotinoid
365 insecticide and acute bee paralysis virus across castes and colonies of black garden ants, *Lasius niger*
366 (Hymenoptera: Formicidae). *Scientific Reports*, 11(1), 1-14. <https://doi.org/10.1038/s41598-021-98406-w>
- 367 Schläppi, D., Kettler, N., Straub, L., Glauser, G., & Neumann, P. (2020). Long-term effects of neonicotinoid
368 insecticides on ants. *Communications Biology*, 3(1), 335. <https://doi.org/10.1038/s42003-020-1066-2>
- 369 Schläppi, D., Stroeymeyt, N., & Neumann, P. (2021). Unintentional effects of neonicotinoids in ants (Hymenoptera:
370 Formicidae). *Myrmecological News*, 31, 181-184. https://doi.org/10.25849/myrmecol.news_031:181
- 371 Schwenke, R. A., Lazzaro, B. P., & Wolfner, M. F. (2016). Reproduction–immunity trade-offs in insects. *Annual*
372 *Review of Entomology*, 61, 239-256. <https://doi.org/10.1146/annurev-ento-010715-023924>
- 373 Siviter, H., Bailes, E. J., Martin, C. D., Oliver, T. R., Koricheva, J., Leadbeater, E., & Brown, M. J. (2021).
374 Agrochemicals interact synergistically to increase bee mortality. *Nature*, 569, pages 389–392.
375 <https://doi.org/10.1038/s41586-021-03787-7>
- 376 Siviter, H., & Muth, F. (2020). Do novel insecticides pose a threat to beneficial insects? *Proceedings of the Royal*
377 *Society B*, 287(1935), 20201265. <https://doi.org/10.1098/rspb.2020.1265>
- 378 Siviter, H., & Muth, F. (2022). Exposure to the novel insecticide flupyradifurone impairs bumblebee feeding
379 motivation, learning, and memory retention. *Environmental Pollution*, 307, 119575.
380 <https://doi.org/10.1016/j.envpol.2022.119575>
- 381 Stanley, J., & Preetha, G. (2016). *Pesticide toxicity to non-target organisms*. Springer. [https://doi.org/10.1007/978-](https://doi.org/10.1007/978-94-017-7752-0)
382 [94-017-7752-0](https://doi.org/10.1007/978-94-017-7752-0)
- 383 Straub, L., Williams, G. R., Pettis, J., Fries, I., & Neumann, P. (2015). Superorganism resilience: Eusociality and
384 susceptibility of ecosystem service providing insects to stressors. *Current Opinion in Insect Science*, 12,
385 109-112. <https://doi.org/10.1016/j.cois.2015.10.010>
- 386 Stroeymeyt, N., Grasse, A. V., Crespi, A., Mersch, D. P., Cremer, S., & Keller, L. (2018). Social network plasticity
387 decreases disease transmission in a eusocial insect. *Science*, 362(6417), 941-945.
388 <https://doi.org/10.1126/science.aat4793>
- 389 Sánchez-Bayo, F., Goulson, D., Pennacchio, F., Nazzi, F., Goka, K., & Desneux, N. (2016). Are bee diseases linked
390 to pesticides?—A brief review. *Environment International*, 89, 7-11.
391 <https://doi.org/10.1016/j.envint.2016.01.009>
- 392 Sánchez-Bayo, F., & Tennekes, H. A. (2020). Time-Cumulative Toxicity of Neonicotinoids: Experimental Evidence
393 and Implications for Environmental Risk Assessments. *International Journal of Environmental Research*
394 *and Public Health*, 17(5), 1629. <https://doi.org/10.3390/ijerph17051629>
- 395 Sánchez-Bayo, F., & Wyckhuys, K. A. (2019). Worldwide decline of the entomofauna: A review of its drivers.
396 *Biological Conservation*, 232, 8-27. <https://doi.org/10.1016/j.biocon.2019.01.020>

- 397 T O'Neal, S., Anderson, T. D., & Wu-Smart, J. Y. (2018). Interactions between pesticides and pathogen
398 susceptibility in honey bees. *Current Opinion in Insect Science*, 26, 57-62.
399 <https://doi.org/10.1016/j.cois.2018.01.006>
- 400 Team, R. C. (2020). *R: A language and environment for statistical computing*. In (Version R version 3.6.3 (2020-
401 02-09)) R Foundation for Statistical Computing. <https://www.R-project.org/>
- 402 Therneau, T. M. (2022a). *A Package for Survival Analysis in R*. R package version 3.4-0. [https://CRAN.R-](https://CRAN.R-project.org/package=survival)
403 [project.org/package=survival](https://CRAN.R-project.org/package=survival)
- 404 Therneau, T. M. (2022b). *coxme: Mixed Effects Cox Models*. R package version 2.2-18.1, [https://CRAN.R-](https://CRAN.R-project.org/package=coxme)
405 [project.org/package=coxme](https://CRAN.R-project.org/package=coxme).
- 406 Tomizawa, M., & Casida, J. E. (2005). Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annual*
407 *Review of Pharmacology and Toxicology*, 45, 247-268.
- 408 Tosi, S., Nieh, J. C., Brandt, A., Colli, M., Fourrier, J., Giffard, H., . . . Simon-Delso, N. (2021). Long-term field-
409 realistic exposure to a next-generation pesticide, flupyradifurone, impairs honey bee behaviour and survival.
410 *Communications Biology*, 4(1), 805. <https://doi.org/10.1038/s42003-021-02336-2>
- 411 Tosi, S., Nieh, J. C., Sgolastra, F., Cabbri, R., & Medrzycki, P. (2017). Neonicotinoid pesticides and nutritional
412 stress synergistically reduce survival in honey bees. *Proceedings of the Royal Society B: Biological*
413 *Sciences*, 284(1869), 20171711. <https://doi.org/10.1098/rspb.2017.1711>
- 414 Wagner, D. L. (2020). Insect declines in the Anthropocene. *Annual Review of Entomology*, 65, 457-480.
415 <https://doi.org/10.1146/annurev-ento-011019-025151>
- 416 Wood, T. J., & Goulson, D. (2017). The environmental risks of neonicotinoid pesticides: a review of the evidence
417 post 2013. *Environmental Science and Pollution Research*, 24(21), 17285-17325.
418 <https://doi.org/10.1007/s11356-017-9240-x>
- 419 Wu, Y.-Y., Pasberg, P., Diao, Q.-Y., & Nieh, J. C. (2021). Flupyradifurone reduces nectar consumption and foraging
420 but does not alter honey bee recruitment dancing. *Ecotoxicology and Environmental Safety*, 207, 111268.
- 421 Zóltowska, K., Fraczek, R., & Lipinski, Z. (2011). Hydrolases of developing worker brood and newly emerged
422 worker of *Apis mellifera carnica*. *Journal of Apicultural Science*, 51(1), 27-37.
423 <https://doi.org/10.1673/031.012.2201>
424

425 **Supplementary Materials**

426 *Antifungal assay: Metarhizium brunneum* growth inhibition test

427 Flupyradifurone specifically impairs the nervous system of insects (Nauen et al., 2015) and thus, no
428 antifungal activity is to be expected from this agrochemical. Nonetheless, any results of combined exposure
429 treatments would be invalidated or at least difficult to interpret if flupyradifurone inhibits the growth of *M.*
430 *brunneum*. To test the ability of flupyradifurone to inhibit *M. brunneum* germination we used a disk-diffusion
431 assay as previously described in Blaouiri et al. (2016). Briefly, SDA plates were inoculated with 50 μ L of
432 10^9 /mL *M. brunneum* conidiospore suspension. Then, filter paper disks (6 mm, Cytiva Life Sciences) were
433 soaked with 15 μ L of flupyradifurone solutions (10, 100, 1000 ppm), distilled water (0 ppm; negative control)
434 or 5% sodium hypochlorite (positive control) and put on the agar using forceps. Plates were sealed, incubated
435 for 48h at 24°C and photographed to measure the growth inhibition diameter. Even at the highest
436 flupyradifurone dose no growth inhibition zone was visible suggesting that *M. brunneum* germinated normally.
437 Our findings are in line with findings for neonicotinoids, which have been used successfully in studies with
438 fungal pathogens where no negative effects on conidia germination, conidia production and vegetative growth
439 of *Metarhizium* fungi were detected (Cramer, 2020; Neves et al., 2001, Santos et al., 2007).



440
441 **Supplementary Figure 1. Antifungal assay.** Disk diffusion method to test for germination inhibition of (a)
442 flupyradifurone at 0, 10, 100 and 1000 ppm and (b) sodium hypochlorite (positive control).

443 Cramer, L. *The synergistic effect of pesticides on the fitness of the ant species Cardiocondyla obscurior*. Master thesis,
444 Karl-Franzens-Universität Graz, (2020).

445 Balouiri, M., Sadiki, M., & Ibsouda, S. K. *Methods for in vitro evaluating antimicrobial activity: A review*. *Journal of*
446 *pharmaceutical analysis*, 6(2), 71-79, doi:10.1016/j.jpha.2015.11.005 (2016).

447 Nauen, R., Jeschke, P., Velten, R., Beck, M. E., Ebbinghaus-Kintscher, U., Thielert, W., . . . Raupach, G.
448 *Flupyradifurone: a brief profile of a new butenolide insecticide*. *Pest Management Science*, 71(6), 850-862
449 (2015).

450 Neves, P. M., Hirose, E., Tchujo, P. T. & Moino JR, A. *Compatibility of entomopathogenic fungi with neonicotinoid*
451 *insecticides*. *Neotropical Entomology* 30, 263-268, doi:10.1590/S1519-566X2001000200009 (2001).

452 Santos, A. V., de Oliveira, B. L. & Samuels, R. I. Selection of entomopathogenic fungi for use in combination with sub-
453 lethal doses of imidacloprid: perspectives for the control of the leaf-cutting ant *Atta sexdensrubropilosa* Forel
454 (*Hymenoptera: Formicidae*). *Mycopathologia* 163, 233-240, doi:10.1007/s11046-007-9009-8 (2007).