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

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Abstract

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

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

Introduction

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

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

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

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

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

Methods

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

Flupyradifurone susceptibility test

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

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

Meal size quantification

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

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

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

Flupyradifurone and fungus co-exposure

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

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

Feeding solutions were freshly prepared and provided every 3 days as described above. After ten days, workers of each petri dish were randomly selected and split into two groups of twenty, one assigned to a fungus exposure and the other to the sham treatment, resulting in a total of six treatment groups. Fungal exposures were performed using standard procedures for the generalist entomopathogen *M. brunneum*, strain MA275, KVL 03-143 (Stroeymeyt et al., 2018). *M. brunneum* was cultured on Sabouraud-Dextrose Agar (SDA) plates at 24°C for 3 weeks or until sporulation was observed. Conidiospores were harvested in 0.05% Triton-X100, washed twice and adjusted to 10^9 spores/mL in 0.05% Triton-X100 to create a spore stock suspension. For the exposure, the stock suspension was diluted by half with 0.05% Triton-X100 resulting in a final spore concentration of $5x10^8$. Individuals were placed on ice to reduce their movement and then 0.3 µL of the spore solution was topically pipetted onto their gaster. Sham treated ants were exposed with the same volume of 0.05% Triton X-100 only. They were left to dry for 3 minutes and then transferred individually into new petri dishes. The order of exposure was organized in a stratified random way to minimize time and cohort effects between treatment groups, as well as being split over 2 blocks on consecutive days. Petri dishes ($\varnothing = 35$ mm, N=600) were prepared in advance with fluon coating and a water tube (200 µL Eppendorf tubes fastened with Blu Tack and closed with a cotton ball). These petri dishes were then sealed with parafilm and placed in the incubator. Survival was then checked daily for two weeks by counting and removing dead ants; the experimenters were blind to treatment during all survival checks.

Statistical Analyses

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

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

Results

Susceptibility test

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

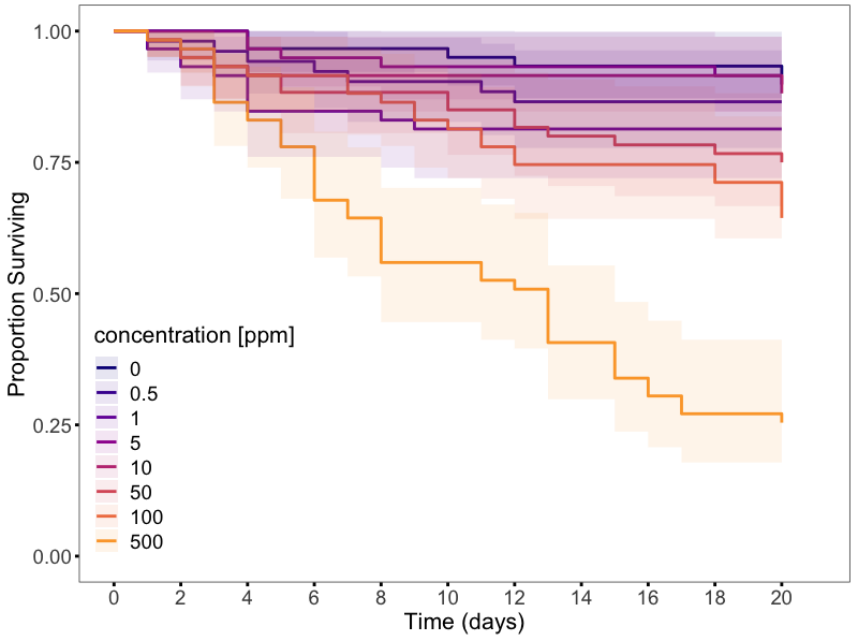

Figure 1: Flupyradifurone susceptibility. Lines represent the proportion of *L. niger* workers surviving as a function of time during chronical exposure to different concentrations of FPF (total sample size: N=467). Shaded areas represent the 95% confidence interval around each line. Concentration had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

Meal size quantification

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

Flupyradifurone and fungus co-exposure

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

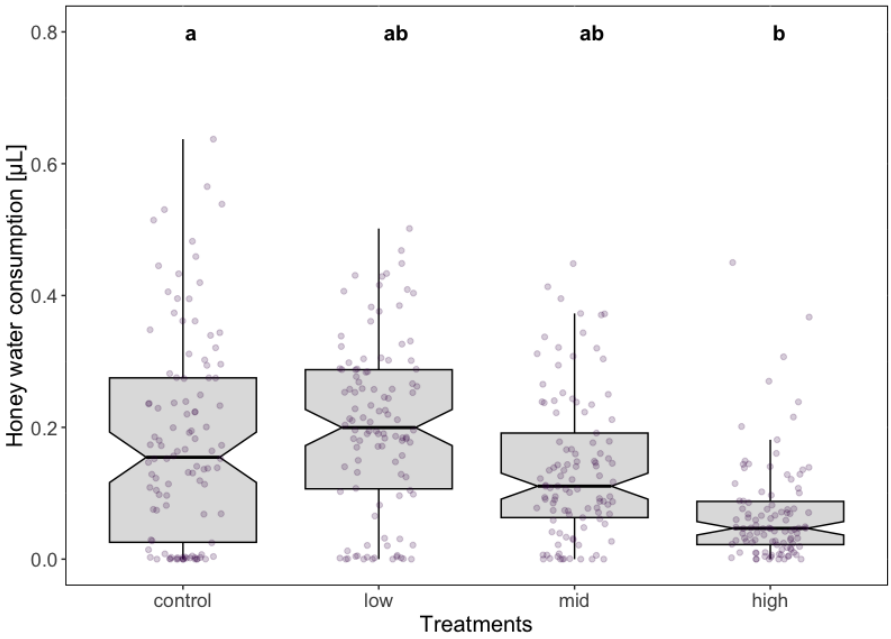

Figure 2: Meal size. Volume (µL) of sugar water consumed by *Lasius niger* workers in 24h at different concentrations of FPF (control = 0, low = 5, mid = 50, high = 500 ppm). Boxplots are shown with the inter-quartile-ranges (box), medians (black line in box) and outliers (dots). Transparent dots represent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

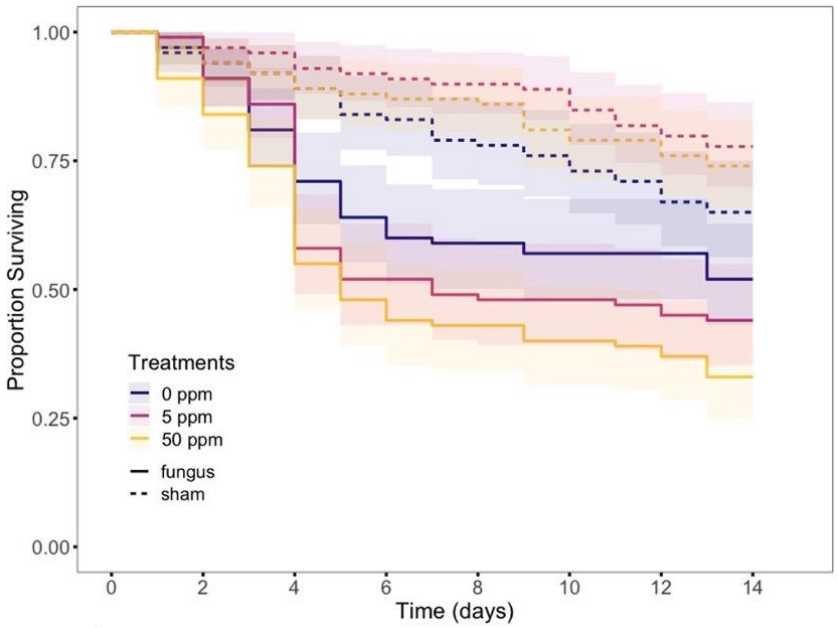

Figure 3: Interactive effects between Flupyradifurone and *M. brunneum*. Lines represent the proportion of *L. niger* workers surviving as a function of time following exposure to live *M. brunneum* spores (solid lines) or to a sham solution (dashed lines) after ten days chronic exposure to different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

Discussion

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

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

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

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

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

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

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

Data availability

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

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Author Contributions

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

Competing Interests statement

The authors declare no conflict of interest.

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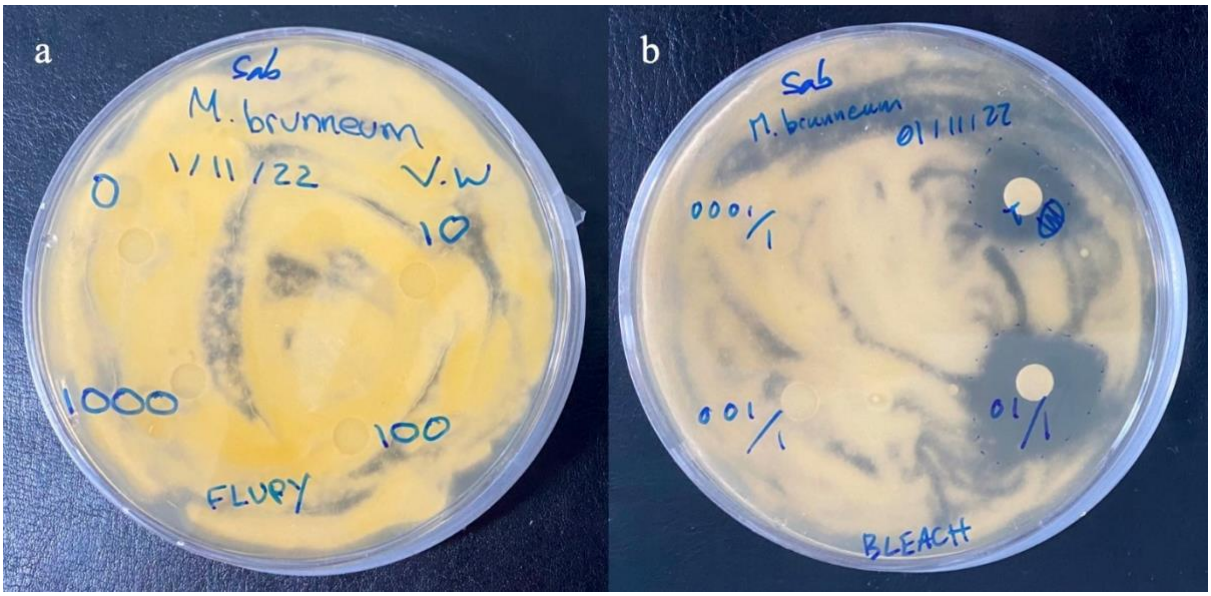
Supplementary Materials

Antifungal assay: Metarhizium brunneum growth inhibition test

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



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

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