ORIGINAL ARTICLE

Effects of sub-nanogram doses of acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor on courtship, mating, and olfactory host finding of the parasitic wasp *Lariophagus distinguendus*

Nils Schöfer | **Gabriel Ratschmann** | **Joachim Ruther** 🄳

Institute of Zoology, University of
Regensburg, Regensburg, Germany

Correspondence
Joachim Ruther, Institute of Zoology,
University of Regensburg, Universitätsstraße
31, 93053 Regensburg, Germany.
Email: joachim.ruther@ur.de

Abstract

Continued use and development of new insecticides are seen as a necessary part of modern agriculture, but insecticides, even at sublethal levels, may also affect beneficial non-target species, such as pollinators, predators, and parasitic wasps. Here, we studied sublethal effects of four insecticides – acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor – all targeting cholinergic neurons in insects, on sexual communication and olfactory host finding in the parasitic wasp *Lariophagus distinguendus* (Förster) (Hymenoptera: Pteromalidae). We found that all four insecticides reduced, at least at one of the tested sublethal doses, pheromone-mediated courtship behaviour (wing fanning) in males and the mating frequency of treated wasps. Treated females no longer preferred host-associated kairomones. This suggests that the olfactory sense of the wasps is hampered by sublethal insecticide doses. The lowest bioactive doses tested in the bioassays were 0.021 ng for sulfoxaflor and 0.105 ng for acetamiprid, dimethoate, and flupyradifurone. These low amounts are field realistic and might be taken up by the wasps, e.g., by feeding contaminated plant nectar. Exposure to these compounds likely compromises the important ecosystem service provided by parasitic wasps as natural enemies in terrestrial ecosystems.

KEYWORDS

chemical communication, ecosystem service, host finding, Hymenoptera, insecticides, natural enemies, non-target species, olfaction, Pteromalidae, sexual communication, sublethal effects, wing fanning

INTRODUCTION

Insect pests are a global threat to agriculture and thus to humanity's food supply. In industrialised agriculture, insect pests have long been controlled with different classes of insecticides, including insect growth regulators, designed to inhibit their growth, reproduction, and/or spread, often by attacking the pests' nervous system (Tomizawa et al., 2003; Watson et al., 2011; Nauen et al., 2015; Van Scoy et al., 2016). These insecticides are easy to use and can be either sprayed in crops or applied to the crop substrate aiming at systemic activity. Due to their non-specificity as well as adverse effects at sublethal levels (Stapel et al., 2000; Demirci & Gungordu, 2020; Huang et al., 2022), insecticides are thought, among other factors, to contribute to the massive decline of insect biodiversity worldwide (Gibbs et al., 2009; Gilburn et al., 2015). There is, however, increasing evidence that very low doses of chemical stressors such as insecticides may also have stimulatory (hormetic) effects on insects (Cutler et al., 2022; Guedes et al., 2022). Many of the active compounds in commercial insecticides are neurotoxins. Some of the most widely used insecticides such as the neonicotinoids overstimulate the nervous system

by attacking nicotinic acetylcholine receptors (nAcChR). The use of these insecticides is considered safe for crops, posing less risk to mammals and humans due to their lower affinity for mammalian nAcChR (Nauen et al., 2015; Van Scoy et al., 2016). However, they still exert pressure on wild non-target insect populations that are even remotely associated with farmland ecosystems (Hallmann et al., 2017; Watson et al., 2011). In addition to being lethal for agricultural insect pests, several insecticides have adverse lethal and sublethal effects on non-target organisms (Fischer et al., 1997; Tosi & Nieh, 2019). The honeybee, in particular, has become an important model organism for studying these insecticide side effects (Colin et al., 2004; Tosi & Nieh, 2019).

Adverse effects on bees were also the reason that three members of the intensively used insecticide class of neonicotinoids – imidacloprid, thiamethoxam, and clothianidin – were banned within the European Union (EU Commision, 2013). Outside the EU, these substances are still widely used, with large markets, for instance in India and China (Wang et al., 2020; Anand et al., 2021). Following this ban, alternative insecticides became more popular within the EU, including other neonicotinoids such as acetamiprid (Jerez et al., 2022). Although novel insecticides such as the butenolide flupyradifurone and the sulfoximine sulfoxaflor have a lower acute toxicity than imidacloprid (Watson et al., 2011; Nauen et al., 2015), they are still effective as control agents against pests. Another mode of action of pesticides found in the organophosphates is the inhibition of the enzyme acetylcholine esterase (O'Brien, 1963). Inhibition of this enzyme prevents degradation of the natural receptor ligand acetylcholine, which leads to overstimulation of the receptor and eventually to paralysis and death in invertebrates. A member of this class is dimethoate, which is no longer approved in the EU but still in use in the USA and many developing markets (Centers for Disease Control and Prevention, 2017).

In the past decades, many insect taxa, including pests (Guedes et al., 2016), pathogen vectors (Agathokleous et al., 2023), and beneficial non-target insects (Desneux et al., 2007), have been studied with respect to sublethal effects of insecticides. Apart from pollinators, parasitic wasps were of particular interest in studies on beneficial non-target species in the past (Desneux et al., 2007; Tappert et al., 2017; Jiang et al., 2019a,b; Kremer & King, 2019; Teder & Knapp, 2019) because of their important function as biocontrol agents of insect pests (Niedermayer et al., 2016). Parasitic wasps have a close relationship with their hosts, developing within or attached to their host species (Godfray, 2010; Niedermayer et al., 2016), many of which are pest insects. As such, these wasps provide a valuable ecosystem service (Bengtsson, 2015). Parasitic wasps are exposed to various insecticides via their use of floral and extrafloral nectar and honeydew as carbohydrate sources (Bugg et al., 1989; Idris & Grafius, 1995; Wäckers et al., 2008; König et al., 2015a; Bailey et al., 2009).

Parasitic wasps, like pollinators, use their olfactory sense for orientation. In particular, they rely on pheromones, kairomones, and synomones to find sexual partners and hosts for egg-laying (Steidle & Schöller, 1997; Zhang et al., 1998; Ruther et al., 2009; Schurmann et al., 2009). As nAcChR are involved in the processing of chemical information in insects (Jones & Sattelle, 2010; Dupuis et al., 2012), previous research on parasitic wasps has shown that insecticides targeting the cholinergic system have sublethal effects on the response of parasitoids to semiochemicals (Tappert et al., 2017; Jiang et al., 2019b; Teder & Knapp, 2019; Schöfer et al., 2023). In the pteromalid wasp *Nasonia vitripennis* (Walker), the neonicotinoid imidacloprid affected the wasps' ability to find and recognize mates, the copulation rate, and their ability to find hosts using host-associated volatiles (Tappert et al., 2017). Similar effects were found in a recent study investigating the effects of acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor on sexual communication, mating, and host finding in *N. vitripennis* (Schöfer et al., 2023). It must be assumed that sublethal effects, such as reported for *N. vitripennis*, have a negative impact on the fitness of wild parasitic wasp populations. As parts of the olfactory system are highly conserved within insects (Couto et al., 2017; Krieger et al., 2003), effects seen within *N. vitripennis* can reasonably be expected to occur also in other parasitic wasps, though the strength of the effects may vary. If the results are transferable to other species, this would certainly affect the efficacy of parasitic wasps as biocontrol agents. Before broader conclusions can be drawn, however, additional species with well-developed bioassay methods need to be studied with respect to their susceptibility to sublethal insecticide doses. Here, we investigate sublethal effects of four insecticides on *Lariophagus distinguendus* (Förster) (Hymenoptera: Pteromalidae), like *N. vitripennis* a member of the subfamily Pteromalinae (Graham, 1969).

Lariophagus distinguendus is a solitary parasitoid wasp that lays its eggs on the late larval to pupal stages of stored-product infesting beetles (Niedermayer et al., 2016). Females find seeds infested by grain weevils, their main hosts, by volatile odorants in the larval faeces (Steidle & Schöller, 1997) and typically lay a single egg per infested seed, though superparasitism may occasionally occur (Bellows, 1985). The hatched larvae feed on the hosts and, depending on the temperature, develop within 14–31 days to the adult stage (Ryoo et al., 1991). Males emerge earlier than females and mating occurs primarily at their natal host patch (Niedermayer et al., 2016). *Lariophagus distinguendus* has shown great potential in controlling populations of many beetle species harmful to grain crops and has been used in this context as part of integrated pest management (Adarkwah et al., 2012; Hansen et al., 2013; Iturralde-Garcia et al., 2020; Harush et al., 2021).

In this paper, we investigate the sublethal effects of acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor applied topically as an acetone solution to the wasps, on the males' response to the female sex pheromone, the

mating frequency, and the females' olfactory host finding ability.

MATERIALS AND METHODS

Insects

A culture of *L. distinguendus* was obtained from a commercial supplier (Biologische Beratung Prozell & Schöller, Berlin, Germany) and reared at 25 °C, 50% r.h., and L16:D8 photoperiod on larvae and pre-pupae of the granary weevil, *Sitophilus granarius* L. (Coleoptera: Curculionidae). Hosts were reared in 400-mL screw-top glass jars containing 200 mL of wheat, *Triticum aestivum* L., grain that had been moistened with 7 mL of distilled water. An amount of 25 mL of newly emerged, mixed sex weevils was added to each glass jar and allowed to mate and lay eggs for 1 week. Subsequently, weevils were removed by sieving and transferred to new breeding jars. On the first Monday of each month, weevils were replaced by those from the next generation. To rear *L. distinguendus*, amounts of ca. 20 g of 4–5-week-old weevil-infested grains were filled into Petri dishes, and ca. 50–100 adult *L. distinguendus* were added. Females were allowed to parasitise weevil larvae until they died. At rearing conditions, the next wasp generation emerged after approximately 21 days. To obtain wasps of defined age and mating status for experimentation, weevil-infested grains parasitised by *L. distinguendus* were individually placed in Eppendorf tubes shortly before their expected emergence. Tubes were examined daily for newly emerged wasps that were then used for the experiments.

Insecticides

Analytical standards of the tested insecticides acetamiprid (≥98.0% purity), dimethoate (≥98.0% purity), and flupyradifurone (≥98.0% purity) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Sulfoxaflor (99.23% purity) was obtained from Dr. Ehrenstorfer (Augsburg, Germany). Insecticides were dissolved in acetone (Rotisolv, ≥99.8% purity; Carl Roth, Karlsruhe, Germany).

Toxicity tests

Testing sublethal effects of substances requires an accurate assessment of the substances' acute toxicity. For this purpose, dilution series (Table S1) of acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor were prepared in acetone, starting with a stock solution of 1 mg mL^{-1}. Aliquots of 210 nL of these dilutions were then applied to the abdomens of ice-cooled wasps using a microinjector (Nanoliter 2010; World Precision Instruments, Sarasota, FL, USA). Topical application was chosen instead of oral uptake to enable exact dosage control (Tappert et al., 2017). The applied volume of acetone has been shown to have no adverse effects in another pteromalid wasp of comparable size (Jatsch & Ruther, 2021). Groups of eight wasps (n = 3 replicates per dose/sex) were treated with ever-decreasing insecticide doses and with pure acetone (control). After 72 h, the proportion of surviving wasps was determined for each insecticide concentration. Survival data were used to fit probit sigmoid dose–response curves for the four insecticides (Figure 1) using MedCal (v.22.016) scientific software (MedCal Software, Ostend, Belgium) and to calculate the median lethal doses (LD50), i.e., those doses (with 95% confidence intervals) from the respective models at which 50% of the treated wasps died. For the bioassays, 2–3 doses were tested for each insecticide that were lethal for <30% of the treated wasps (Table 1). For control, wasps treated with pure acetone were used in the bioassays.

Effects on pheromone communication

Male *L. distinguendus* show a characteristic wing-fanning behaviour when perceiving the odour of a female, and this behaviour is an integral part of the mating ritual (Benelli et al., 2014; Ruther et al., 2000). Assessing whether the four insecticides inhibit this response, males (n = 20 for each experiment) were treated with a dose of the active ingredient or with pure acetone (control) and placed 1 day later into a round mating arena (2 cm diameter, 5 mm height) containing a dead female (dummy). Dummies were prepared by freezing females at −20 °C shortly after emergence (maximum age 1 day). Previous studies demonstrated that males react strongly to these dummies and exhibit wing fanning in response to the female contact pheromone consisting of cuticular hydrocarbons (Steiner et al., 2005; Kühbandner et al., 2013). Preliminary experiments revealed that untreated males and control males treated with acetone show no significant difference in their reactions (results not shown). Males were exposed to the female dummies for 5 min with the number of wing-fanning bouts being recorded using The Observer XT 15 software (Noldus Information Technology, Wageningen, The Netherlands). Dummies were used only once, and the arena was cleaned with ethanol after each bioassay.

Effects on mating frequency

Male and female *L. distinguendus* exhibit ritualized courtship behaviour that involves the female contact sex pheromone arresting males near the female (Ruther et al., 2000) and a male oral pheromone of unknown chemical structure that males spread over the female
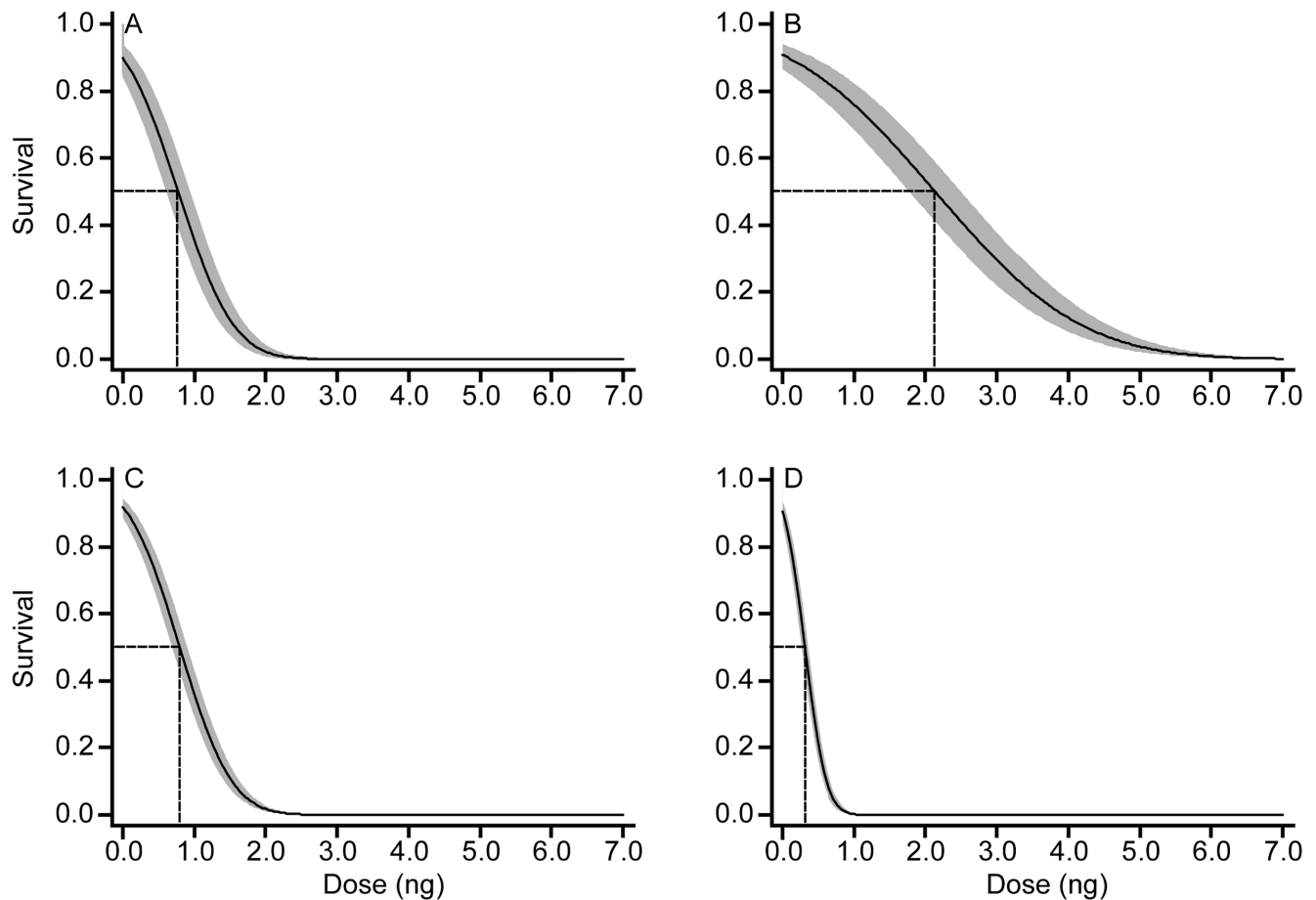
FIGURE 1 Dose–mortality curves after 72 h of *Lariophagus distinguendus* treated topically with various doses of (A) acetamiprid, (B) dimethoate, (C) flupyradifurone, and (D) sulfoxaflor dissolved in acetone. The shaded area indicates the 95% confidence interval, the dashed lines the median lethal doses (LD50).

TABLE 1 LD50-values (evaluated after 72 h) and 95% confidence intervals (CI) obtained from the toxicity tests by fitting probit sigmoid dose–response curves (see Figure 1) using MedCal (v.22.016) scientific software, and doses of the four insecticides tested in the bioassays with *Lariophagus distinguendus*. In parentheses the percentages of surviving wasps in the toxicity tests. Sublethal doses, i.e., those below the no-observed-effect-level (NOEL, see Table S1) are indicated by an octothorpe (#). Doses indicated with 'n.t.' were tested in some of the bioassays but not in the toxicity tests.

	LD50 (ng/wasp)	95% CI	Tested (sublethal) doses (ng/wasp)		
Acetamiprid	0.77	0.63–0.91	0 (96%)	0.105# (94%)	0.21# (83%)	0.42 (n.t.)
Dimethoate	2.1	1.8–2.5	0 (90%)	0.105# (88%)	0.21# (96%)	0.63# (92%)
Flupyradifurone	0.80	0.70–0.90	0 (96%)	0.105# (n.t.)	0.21# (83%)	0.42 (77%)
Sulfoxaflor	0.30	0.26–0.36	0 (92%)	0.021# (94%)	0.063# (92%)	0.105# (83%)

antennae to elicit receptivity (König et al., 2015b). For this series of experiments, wasp pairs were assigned to three groups. In the first group, only the males were treated 1 day prior to the bioassay with a sublethal dose of the insecticides. In the second group, only the females and in the third group both partners were treated (n = 20 for each treatment). For the bioassay, the pair was placed into a courtship arena of the same dimensions as the one used in the pheromone experiments, with the female always being placed first. Once the male was added, the arena was closed with a cover slip, and the pair was observed for 5 min using The Observer XT 15 software. If the female accepted the male and allowed copulation, the

trial was recorded as a success. For control, the respective mates were treated with pure acetone.

Effects on host finding

After mating, female *L. distinguendus* search for hosts by using volatiles emitted from larval host faeces (Steidle & Schöller, 1997; Steiner et al., 2007). To test sublethal effects of the four insecticides on this process, mated females were treated with a sublethal insecticide dose (control: acetone; n = 20 for each treatment). After the treatment, females were kept overnight in Eppendorf

tubes with five weevil-infested wheat grains. The lids of the tubes were punctured with a fine needle to supply the wasps with air. This pre-treatment was performed to increase the responsiveness of females to the odour of host faeces, as they have been shown to learn odours associatively when rewarded with oviposition success (Steidle, 1998). The next day, the response of females to the odour of host faeces was tested in a four-chamber olfactometer (Steidle & Schöller, 1997; Ruther & Steidle, 2000). This olfactometer consisted of a plastic cylinder (19 cm diameter, 4 cm height) divided by vertical plates into four equal chambers. The cylinder was covered by a walking arena of metal mesh that prevented visual orientation to the samples presented in the chambers but allowed diffusion of volatiles from the samples through the mesh into the walking arena. The walking arena was covered by a glass plate to prevent the wasps from escaping. A cardboard cylinder (21 cm diameter, 9 cm height) was placed over the arena to exclude external visual stimuli, and the olfactometer was illuminated from above by a desk lamp (60 W). In one of the chambers, a glass dish was presented that contained 150 mg of *S. granarius* larval faeces, whereas the opposite chamber (control) contained an empty glass dish. The two remaining chambers of the olfactometer were left empty. A female (n = 20 per treatment) was then placed in a central cavity of the arena (starting point) and allowed to move freely around the arena for 5 min. The residence time of females in the sectors above the sample and the control chamber, respectively, was recorded using the Observer XT 15 software. The olfactometer was rotated 90° clockwise after each observation to avoid biased results due to potential side preferences.

Quantification of artificial nectar consumption

Contaminated carbohydrate sources such as plant nectar are likely natural sources of exposure for parasitic wasps. To evaluate whether the concentrations of insecticides reported in the literature justify the claim that *L. distinguendus* may take up bioactive doses via plant nectar, it is necessary to know the volume of nectar taken up by the wasps. Therefore, a feeding experiment with artificial nectar (30% glucose in water) was performed as recently described for *N. vitripennis* (Schöfer et al., 2023). Briefly, females (n = 6) were exposed for 48 h in Eppendorf tubes to 10 μL of the glucose solution. After this period, females were removed, and the remaining glucose was quantified after derivatization by gas chromatography/mass spectrometry (GC/MS). Control tubes (n = 6) were treated equally without adding a wasp. The difference between the two treatments gives an estimate of the glucose solution consumed by the wasps. For more details regarding the bioassay, the sugar derivatization method, and the GC/MS parameters see Schöfer et al. (2023).

Statistical analysis

All statistical analyses were performed using R v.4.0.1 (R Development Core Team, 2023). Data from the pheromone experiment were analysed with a Kruskal-Wallis test and subsequent Dunn's tests for comparisons between each sublethal dose and the respective acetone control utilising the kruskal.test (base R package) and dunn.test (dunn.test package) functions. The survival rates of insecticide-treated and control wasps, copulation rates from the courtship experiments, as well as the number of males not showing any wing-fanning behaviour in the pheromone experiments were analysed with Fisher's exact test. Residence times of females in host and control zones in the host finding experiment were compared by Wilcoxon matched pairs test. To visualise the data, PAST v.4.03 software (Hammer et al., 2001) was used to plot bar charts and boxplots.

RESULTS

Toxicity tests

The dose-mortality curves disclosed the insecticides differing strongly in their acute toxicity (Figure 1, Table 1). Dimethoate (model fit: $\chi^2 = 159.77$, df = 1, P < 0.001) exhibited the lowest toxicity (LD50 = 2.1 ng), acetamiprid ($\chi^2 = 220.68$, df = 1, P < 0.001, LD50 = 0.77 ng) and flupyradifurone ($\chi^2 = 274.27$, df = 1, P < 0.001, LD50 = 0.80 ng) displayed intermediate toxicities, whereas sulfoxaflor ($\chi^2 = 354.82$, df = 1, P < 0.001) was most toxic to *L. distinguendus* (LD50 = 0.30 ng). The differences in acute toxicity of the four insecticides led to the use of different sublethal doses in the bioassays depending on the active ingredient (Table 1). Sublethal doses are defined as inducing no statistically significant mortality in an experimental population (Desneux et al., 2007). Accord to this definition, the no-observed-effect levels (NOEL) for acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor were determined as 0.21, 0.63, 0.21, and 0.105 ng, respectively (Tables 1 and S1). Except for the highest doses of acetamiprid and flupyradifurone, the doses tested in this study may be considered as sublethal (Tables 1 and S1).

Effects on pheromone communication

In all experiments, acetone-treated control males exhibited extensive wing-fanning behaviour towards female dummies (Figure 2). Except for the lowest dose of dimethoate, each dose of all four active ingredients reduced the number of wing-fanning bouts in insecticide-treated males. Sulfoxaflor affected male wing fanning at doses as low as 0.021 ng, whereas acetamiprid (≥0.21 ng), flupyradifurone (≥0.105 ng), and dimethoate (≥0.21 ng) showed effects only at the higher of the tested doses, but still in the sub-nanogram range. With all substances, there were also males that no longer showed any wing-fanning behaviour at all. However, when compared to the control males
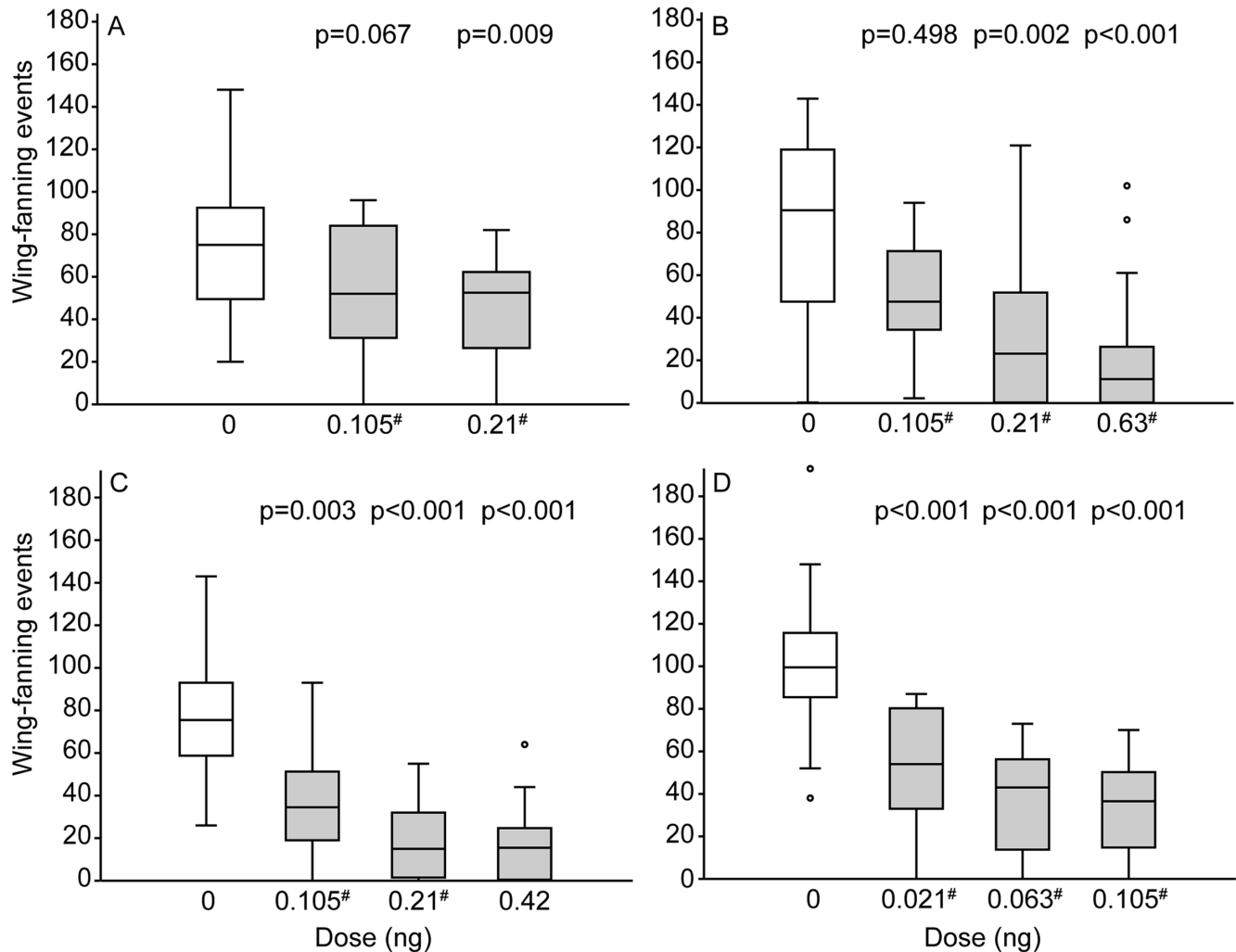
FIGURE 2 Effect of (sublethal) doses (ng) of (A) acetamiprid, (B) dimethoate, (C) flupyradifurone, and (D) sulfoxaflor on the wing-fanning response (no. bouts during 5 min observation) of virgin *Lariophagus distinguendus* males to freeze-killed females (dummies). Control males were treated with pure acetone (dose 0 ng). Box-and-whisker plots show 25 and 75% quartiles (upper and lower end of the boxes), median (horizontal line in between), 1.5× the interquartile range (whiskers), and outliers (dots). The p-values are based on comparison between treatments and the respective control (Kruskal-Wallis test and subsequent Dunn's tests; n = 20). Sublethal doses are indicated by an octothorpe (#).

(100% response), the number of these non-responders was significantly increased only for the two higher doses of flupyradifurone (Fisher's exact test, 0.21 and 0.42 ng: both 75% response, P = 0.047).
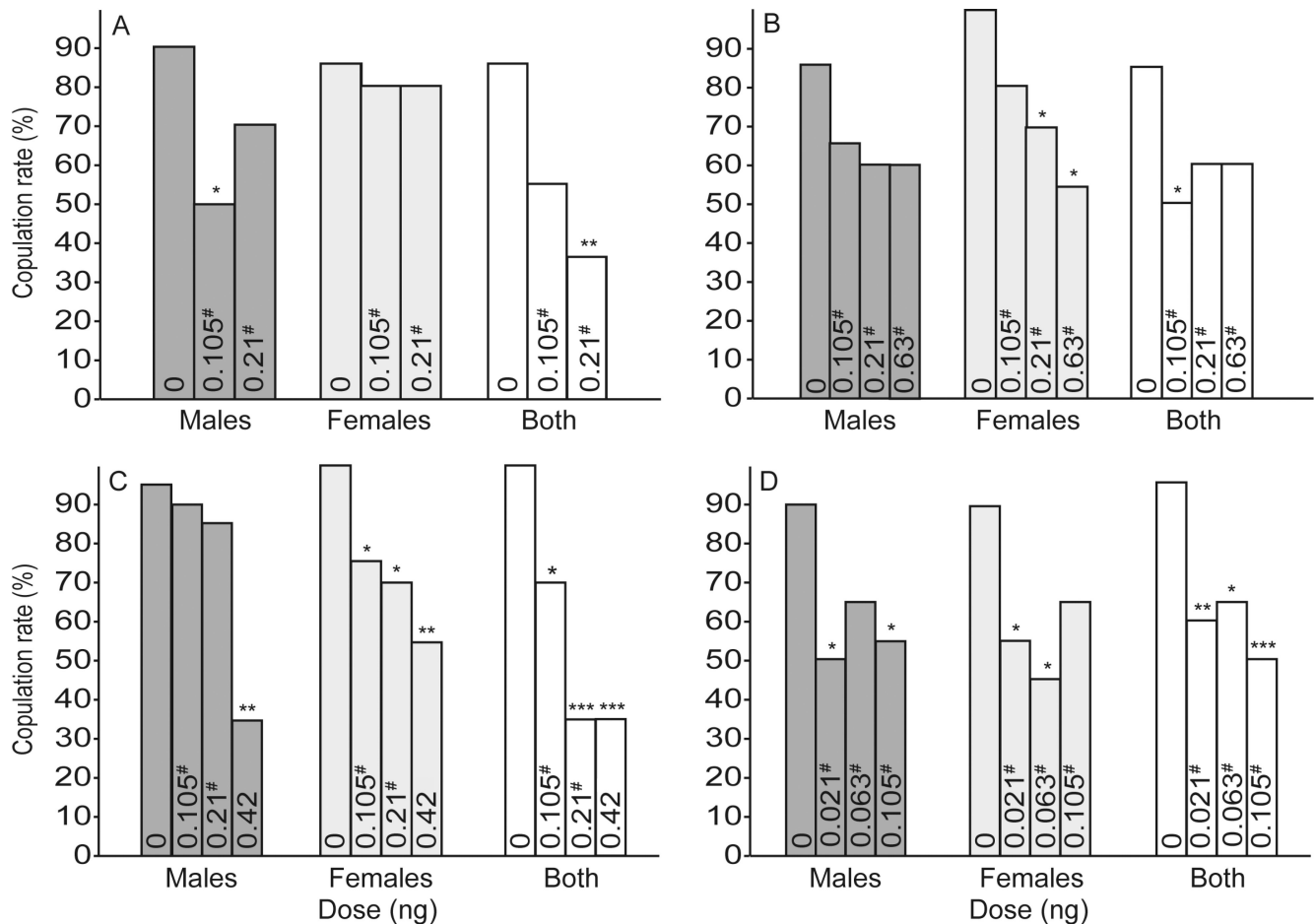
Effects on mating frequency

Copulation rates of control pairs treated with acetone ranged from 85 to 100%. Compared to the controls, each insecticide significantly reduced mating frequencies in at least one treatment constellation or dose (Figure 3). When only males were treated, the highest doses of flupyradifurone and sulfoxaflor and the intermediate doses of acetamiprid, and sulfoxaflor significantly reduced mating frequency (Figure 3A, C, D). Dimethoate had no significant effects in this constellation (Figure 3B). When only females were treated, the higher doses of dimethoate (≥0.21 ng)

and all tested doses of flupyradifurone (≥0.105 ng) decreased the percentage of successful mating (Figure 3B, C). When females were treated with sulfoxaflor, the two lower doses reduced the mating frequency significantly, whereas the reduction was no longer significant at the highest tested dose (Figure 3D). Acetamiprid applied to females did not reduce the mating frequency at the tested dose range. When both mates were treated, however, all insecticides had significant negative effects on mating frequency already at sub-nanogram doses with flupyradifurone causing a decrease from 100 to 35% at doses ≥0.21 ng (Figure 3).

Effects on host finding

In all control experiments, acetone-treated females showed a significant preference for the host zone of the olfactometer (Figure 4). When treated with any of the sublethal doses

FIGURE 3 Effect of (sublethal) doses (ng) of (A) acetamiprid, (B) dimethoate, (C) flupyradifurone, and (D) sulfoxaflor on the mating frequencies of virgin *Lariophagus distinguendus*. Shown are the mating frequencies of pairs, in which either the male, the female, or both partners were treated with pure acetone (0 ng, control) or an insecticide dose (given at the bottom of the columns). Asterisks indicate significant differences between the sublethal doses and the respective control (Fisher's exact test: *0.01 < p < 0.05, **0.001 < p < 0.01, ***p < 0.001; n = 20). Sublethal doses are indicated with an octothorpe (#).

of acetamiprid (≥0.105 ng), females no longer preferred the host odour (Figure 4A). In contrast, dimethoate and sulfoxaflor disrupted the preference for host odour only at the highest tested doses (0.63 ng dimethoate, 0.105 ng sulfoxaflor; Figure 4B, D). Interestingly, females treated with 0.105 ng of flupyradifurone spent similar amounts of time in the host and control zones, whereas those treated with higher doses (0.21–0.42 ng) again showed a slight but significant preference for host odour (Figure 4C).

Quantification of artificial nectar consumption

After the 48-h feeding period, 3.57 ± 0.0.16 mg (mean ± SE) glucose was recovered from the control Eppendorf tubes whereas in those containing *L. distinguendus* females significantly lower amounts were found (2.85 ± 0.17 mg; Mann–Whitney U-test: P = 0.031). Hence, female *L. distinguendus* consumed 20% of the total amount supplied to them (0.72 mg) within 48 h. With a sample volume of 10 μL

used in the feeding experiment, females ingested approximately 2 μL of the artificial nectar.

DISCUSSION

The results of this study expand our understanding of the adverse effects of insecticides on beneficial species by showing that the four insecticides acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor interfere with the function of the olfactory system of *L. distinguendus*. Treatment of the wasps with sublethal doses of the four active substances resulted in wasps struggling to recognise mating partners and hosts by their odours. A comparison of the results of the present study with *N. vitripennis*, for which similar experiments were recently conducted with the same four insecticides (Schöfer et al., 2023) revealed that, except for dimethoate, *L. distinguendus* is much more sensitive to the insecticides than *N. vitripennis* (Table 2). The LD50 of acetamiprid, flupyradifurone, and sulfoxaflor is 16-, 50-, and 128-fold lower for *L. distinguendus*. This
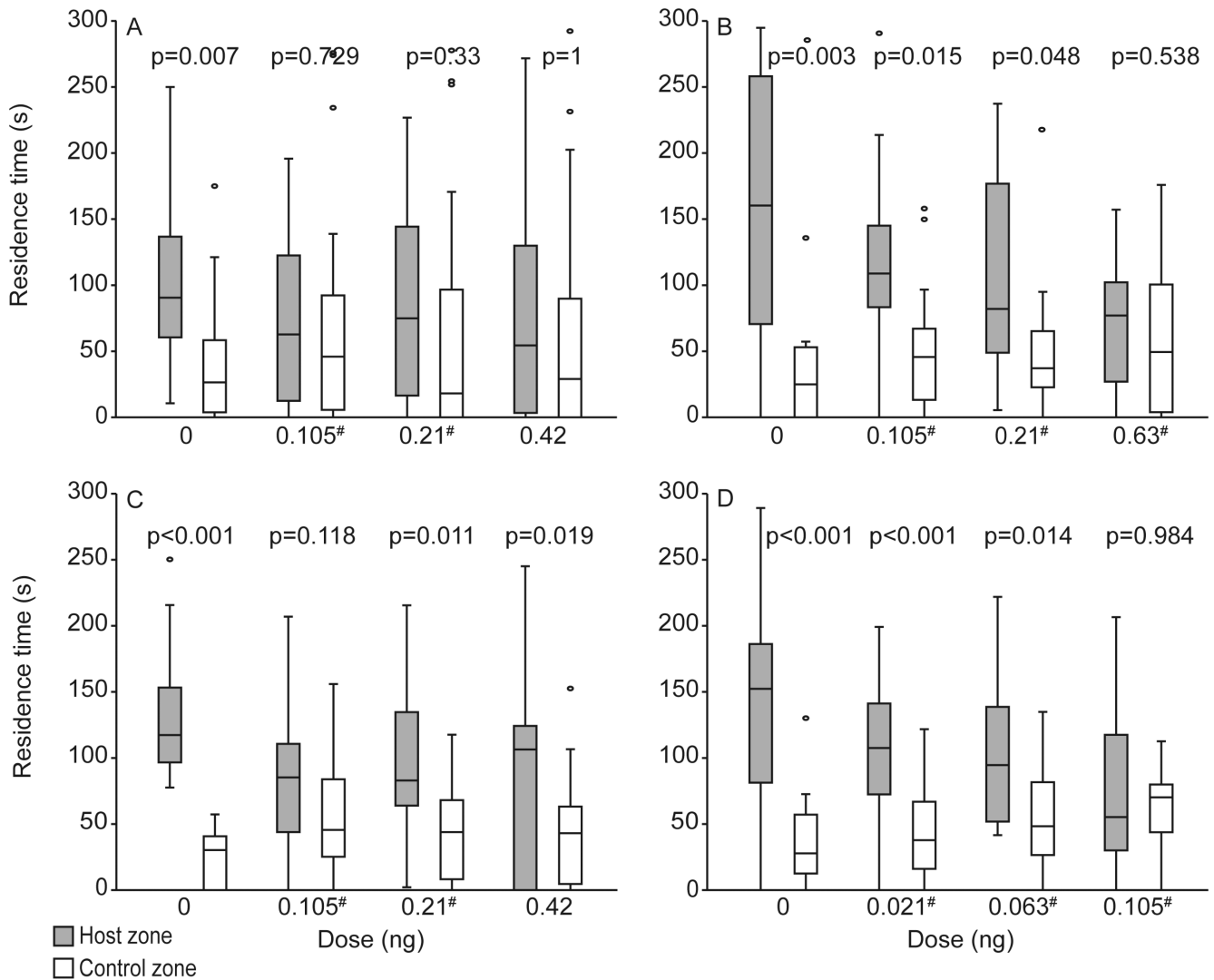
FIGURE 4 Effect of (sublethal) doses (ng) of (A) acetamiprid, (B) dimethoate, (C) flupyradifurone, and (D) sulfoxaflor on the residence time (s) of mated *Lariophagus distinguendus* females in the host zone (grey bars) and the untreated control zone (white bars) of the olfactometer, after exposure to host odour (150 mg larval faeces of *Sitophilus granarius*). Females were either treated with pure acetone (0 ng, control) or an insecticide dose and tested in a four-chamber olfactometer 1 day after application. Box-and-whisker plots show 25 and 75% quartiles (upper and lower end of the boxes), median (horizontal line in between), 1.5× the interquartile range (whiskers), and outliers (dots). The p-values are based on comparison between zones within a treatment (Wilcoxon matched pairs test; n = 20). Sublethal doses are indicated by an octothorpe (#).

difference in acute toxicity was associated with the fact that much lower doses of the three insecticides were sufficient to affect pheromone-mediated mate finding, mating frequency, and olfactory host finding in *L. distinguendus*. For example, doses as low as 21 pg of sulfoxaflor reduced both the wing-fanning response of males to the female sex pheromone and the mating frequency in *L. distinguendus*, whereas in *N. vitripennis* a 125-fold higher dose was required to affect the mating frequency, and the pheromone response of females to the male sex pheromone was not affected at all even at a 250-fold higher dose. Similar stark differences between the two species were observed for flupyradifurone and, less pronounced, for acetamiprid. As both species are closely related (subfamily Pteromalinae) and have a similar size (2–3 mm), this difference in sensitivity is surprising and confirms previous findings in bees. The

leafcutter bee *Megachile rotunda* Fabricius has been shown to be >2500- and 170-fold more sensitive (acute toxicity) to the neonicotinoid thiachloprid and flupyradifurone, respectively, than other managed bee pollinators. This difference is due to *M. rotunda* lacking a P450 enzyme involved in the detoxification of xenobiotics (Hayward et al., 2019). Whether this is also the cause of the difference in sensitivity of *N. vitripennis* and *L. distinguendus* requires further investigation. In any case, the results with *M. rotunda* and those of the present study show that results obtained with one model organism in toxicological studies are not necessarily transferable to others, even closely related ones. This must be taken into account in future approval procedures for new active substances.

Wing-fanning plays an important role in interspecific communication of many parasitic wasps (Benelli

TABLE 2 Comparison between the susceptibility of *Lariophagus distinguendus* (this study) and *Nasonia vitripennis* (Schöfer et al., 2023) to acetamiprid (Ace), dimethoate (Dim), flupyradifurone (Flu), and sulfoxaflor (Sul). Given are the median lethal doses (LD50) and the lowest sublethal doses (ng) that caused significant effects in bioassays studying pheromone response, mating frequency, and olfactory host finding. Factor indicates the relative sensitivity of *L. distinguendus* compared to *N. vitripennis*: more sensitive (>1) or less sensitive (<1).

		N. vitripennis [A]	*L. distinguendus* [B]	Factor [A/B]
LD50 (ng)	Ace	12.6	0.77	16
	Dim	2.2	2.1	1.05
	Flu	102	0.80	128
	Sul	14.9	0.30	50
Pheromone response	Ace	0.63	0.105	6
	Dim	0.105	0.21	0.5
	Flu	21	0.105	200
	Sul	>5.25	0.021	>250
Mating frequency	Ace	6.3	0.105	60
	Dim	1.05	0.105	10
	Flu	21	0.105	200
	Sul	2.63	0.021	125
Host finding	Ace	1.05	0.105	10
	Dim	0.105	0.63	0.17
	Flu	5.25	0.105	50
	Sul	0.525	0.105	5

et al., 2012, 2013b). In *L. distinguendus*, this behaviour is elicited by cuticular hydrocarbons of the female which are processed by the male's olfactory system (Ruther et al., 2000; Benelli et al., 2013a). When compared to acetone-treated control males, each insecticide reduced the number of wing-fanning bouts in reaction to female dummies. The proportion of treated males showing no longer wing fanning at all was significantly increased only for the two highest flupyradifurone doses suggesting that these inactive males were no longer able to perceive the females' cuticular hydrocarbons. In treated males which exhibited wing fanning but to a lesser extent than the control males, motor skill impairment may have contributed to the reduced wing-fanning response in addition to olfactory impairment, as low doses of insecticides targeting nAChR may also influence motor function in insects (Eiri & Nieh, 2012; Williamson et al., 2014). Either way, given that the frequency of wing fanning in *L. distinguendus* differs between successful and unsuccessful males (Benelli et al., 2013a), it is reasonable to assume that insecticide-induced impairment of male wing fanning leads to a reduction in their mating success.

Another decisive stimulus for successful mating in *L. distinguendus* is the male's oral aphrodisiac pheromone (König et al., 2015b). Females that are no longer able to perceive this chemical signal due to insecticide treatment should remain unreceptive. Consistent with these considerations, treating males, females, or both partners with insecticides reduced the mating frequency in our experiments to levels as low as 35% for flupyradifurone. A recent study demonstrated that sublethal doses

of flupyradifurone modified the cuticular hydrocarbon profile in an oligolectic bee (Boff & Ayasse, 2023). In our study, treatment of females with flupyradifurone reduced the mating frequency at any of the tested doses. Given the importance of cuticular hydrocarbons in the courtship behaviour of *L. distinguendus* (Steiner et al., 2005; Kühbandner et al., 2012) and the fact that slight modifications of the profiles can disrupt the male response (Kühbandner et al., 2013), it will be interesting to investigate whether a flupyradifurone-induced change of the female cuticular hydrocarbon profile has contributed to the reduction of the mating frequency and whether other insecticides cause similar effects. A recent study investigating sublethal effects of the pyrethroid λ-cyhalothrin on a leaf beetle suggests that sublethal effects of insecticides on the CHC profile of insects are not restricted to flupyradifurone (Müller et al., 2017).

In the mating experiments, it is noticeable that some findings seem to be contradictory or inconsistent. For example, it is difficult to understand why an insecticide has an effect when only one sex was treated, but this effect is lost when both partners were treated. Also, effects of a low or medium dose that are lost at a higher dose seem difficult to understand at first glance. However, such biphasic dose–response relationships are not uncommon in biology, toxicology, and medicine (Calabrese, 2013; Cutler et al., 2022; Guedes et al., 2022). An alternative explanation is the fact that the mating frequency was not 100% in all control experiments. Hence, only trends were observed in some experiments that might translate into statistically significant differences at higher sample sizes.

Except for parthenogenic insects, mating is a prerequisite to produce offspring. Therefore, it is tempting to assume that the effects of failed mating are less severe in *L. distinguendus* than in other insects, because, as a haplodiploid species, females are able to produce male offspring from unfertilized eggs. However, *L. distinguendus* is a quasi-gregarious parasitoid with a mating system characterized by local mating competition, and the sex ratio is typically skewed towards females when sufficient high-quality hosts are available (Simbolotti et al., 1987; Werren & Simbolotti, 1989). Hence, virginity caused by exposure to sublethal insecticide residues is likely associated with fitness costs for *L. distinguendus* females.

Treatment of *L. distinguendus* females with each of the four insecticides also hampered their ability to orientate toward volatiles present in the larval faeces of their hosts. The ability to use chemical cues from hosts and host-associated materials is key to the reproductive success of most parasitic wasps (Steidle & van Loon, 2002). Previous studies with *Microplitis croceipes* Cresson (Stapel et al., 2000) and *N. vitripennis* (Tappert et al., 2017) revealed similar effects with the neonicotinoid imidacloprid. In *Aphidius ervi* Haliday, the carbamyltriazole triazamate disrupted olfactory host finding, whereas λ-cyhalothrin, the organophosphate chlorpyrifos, and the carbamate pirimicarb showed no effects (Desneux et al., 2004a). Likewise, no effects were observed for the pyrethroid deltamethrin in *Aphidius matricariae* Haliday and *Diaeretiella rapae* M'Intosh (Desneux et al., 2004b). Treatment of the *Drosophila*-parasitoid *Leptopilina heterotoma* Thomson with a LD20 of chlorpyrifos and deltamethrin even improved odour-guided host finding (Delpuech et al., 2005). This indicates that the effects of insecticides on the host-finding process of parasitic wasps may be variable and they need to be investigated with multiple species before more general conclusions on a given substance may be drawn.

The results of our previous study investigating the effects of the four insecticides tested here on *N. vitripennis* (Schöfer et al., 2023) suggested that host and mate finding are affected differently. Sulfoxaflor interfered with females' host finding at all doses tested but had no effect on their response to the male sex pheromone. Dimethoate, on the other hand, disrupted pheromone orientation of *N. vitripennis* females, whereas the preference for host-associated volatiles even turned into avoidance. Similar effects were found in the present study for acetamiprid which had no detectable effects on the mating frequency, i.e., on the females' perception of the male aphrodisiac pheromone, but interfered with host finding at all doses tested. Future studies are needed to unravel the neurophysiological mechanisms underlying the differential effects of sublethal insecticide doses on sexual communication or host finding in parasitic wasps.

In toxicological studies, it is important to discuss whether the results are ecologically relevant. One might argue that *L. distinguendus* is not an ideal model system to study sublethal effects of insecticides in parasitic wasps, as this species is typically found in grain warehouses where it parasitizes larvae of beetles infesting grain and stored products (Niedermayer et al., 2016). The use of fumigants such as methyl bromide and insecticides such as spinosad, methoprene, or chlorpyrifos is common practice in many countries to protect stored grain from pests (Daglish et al., 2018). Therefore, *L. distinguendus* is likely exposed to insecticidal substances in its natural habitat, but, to our knowledge, none of the four insecticides tested here is currently used in stored-product environments. Little is known about the occurrence of *L. distinguendus* outside human grain and food stores, but according to Graham (1969) it is also found in open country, where it might get into contact with contaminated floral and extrafloral nectar, honeydew, or guttation water all of which have been shown to be consumed by parasitic wasps (Jervis et al., 1993; Idris & Grafius, 1995; Röse et al., 2006; Wanner et al., 2006; Urbaneja-Bernat et al., 2020). All these fluids may contain pesticide residues and may thus function as carriers of these substance to parasitic wasps (Davis et al., 1988; Schmolke et al., 2018; Zhou et al., 2022). The doses for topical application used in the present study were chosen according to their acute toxicity (causing <30% mortality) and most of them were in fact sublethal. Topical application is an unnatural way to expose insects to pesticides, but it is standard in toxicological studies and allows more precise control of the dose than offering contaminated food sources. Assuming that topical application and oral uptake of the insecticides have comparable effects and considering the volume of sugar solution ingested by *L. distinguendus* (approximately 2 μL) and the reported residues of the four insecticides in nectar (Table S2), *L. distinguendus* would take up doses in the field that are sufficient to cause adverse effects on mate and host finding. Nonetheless, studies under more realistic conditions are necessary that consider also other routes of uptake such as contact with treated plants or via the host to fully assess the extent of exposure of parasitic wasps to insecticides in the field.

The results obtained in the present study as well as earlier work (Desneux et al., 2007; Tappert et al., 2017; Jiang et al., 2019a,b; Kremer & King, 2019; Teder & Knapp, 2019; Willow et al., 2019; Schöfer et al., 2023) strongly suggest that the ecosystem service provided by parasitic wasps as natural enemies and biocontrol agents is compromised when these are exposed to sublethal residues of insecticides, and there is ample evidence that other insects are affected similarly (Haynes, 1988; Guedes et al., 2016; Desneux et al., 2007; Müller, 2018). Hopefully, these findings will accelerate the shift toward more sustainable crop protection with even more prudent use of pesticides to ensure species-rich and functioning ecosystems in the future.

AUTHOR CONTRIBUTIONS

Nils Schöfer: Formal analysis (lead); investigation (lead); methodology (equal); supervision (supporting); visualization (lead); writing – original draft (equal); writing – review

CONFLICT OF INTEREST STATEMENT
The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT
All data presented in this paper are available online in the supporting information.

ORCID
Joachim Ruther https://orcid.org/0000-0002-1295-347X

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Results of the toxicity tests with *Lariophagus distinguendus* treated with 210 nL each of diluted acetone stock solutions (1 mg/mL) of acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor. Given is the number of live wasps/dead wasps (evaluated 72 h after the treatment). P-values are based on the comparison between pesticide doses and the respective controls (0 ng, pure acetone; Fisher's exact test). Yellow entries indicate no-observed-effect-levels (NOEL). Doses ≤ NOEL are considered as sublethal doses.

Table S2. Literature data on the contamination of floral and extrafloral nectar with the four insecticides tested in this study. Values refer to an assumed consumption of 2 μL (approx. 2 mg) by *Lariophagus distinguendus*. For comparison, the lowest sublethal doses are given having shown significant effects in this study in at least one of the bioassays.

Data S1: Raw data file.