## Environmental Toxicology

# Sublethal Effects of Four Insecticides Targeting Cholinergic Neurons on Partner and Host Finding in the Parasitic Wasp *Nasonia vitripennis*

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**Abstract:** Lethal and sublethal effects of pesticides on nontarget organisms are one of the causes of the current decline of many insect species. However, research in the past decades has focused primarily on pollinators, although other beneficial nontarget organisms such as parasitic wasps may also be affected. We studied the sublethal effects of the four insecticides acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor on pheromone-mediated sexual communication and olfactory host finding of the parasitic wasp *Nasonia vitripennis*. All agents target cholinergic neurons, which are involved in the processing of chemical information by insects. We applied insecticide doses topically and tested the response of treated wasps to sex pheromones and host-associated chemical cues. In addition, we investigated the mating rate of insecticide-treated wasps. The pheromone response of females surviving insecticide treatment was disrupted by acetamiprid ($\geq 0.63$ ng), dimethoate ($\geq 0.105$ ng), and flupyradifurone ($\geq 21$ ng), whereas sulfoxaflor had no significant effects at the tested doses. Olfactory host finding was affected by all insecticides (acetamiprid $\geq 1.05$ ng, dimethoate $\geq 0.105$ ng, flupyradifurone $\geq 5.25$ ng, sulfoxaflor $\geq 0.52$ ng). Remarkably, females treated with $\geq 0.21$ ng dimethoate even avoided host odor. The mating rate of treated *N. vitripennis* couples was decreased by acetamiprid (6.3 ng), flupyradifurone ($\geq 2.63$ ng), and sulfoxaflor (2.63 ng), whereas dimethoate showed only minor effects. Finally, we determined the amount of artificial nectar consumed by *N. vitripennis* females within 48 h. Considering this amount ($\sim 2$ µL) and the maximum concentrations of the insecticides reported in nectar, tested doses can be considered field-realistic. Our results suggest that exposure of parasitic wasps to field-realistic doses of insecticides targeting the cholinergic system reduces their effectiveness as natural enemies by impairing the olfactory sense. *Environ Toxicol Chem* 2023;00:1–12. © 2023 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

**Keywords:** Insecticides; *Nasonia*; Olfaction; Parasitic wasp; Sublethal effects

## INTRODUCTION

As modern agriculture has developed to supply a world of eight billion people, multiple agrochemicals have been established to improve crop yields. Pesticides, chemicals designed to control animals and plants that damage crops, are widely used but also controversial because many substances have been shown to be harmful to natural ecosystems (Sánchez-Bayo, 2021; Uhl & Brühl, 2019). In particular, insecticides such as neonicotinoids are considered a cause of the massive pollinator declines in western Europe (Pistorius et al., 2010). In addition to their intended lethal effects on target organisms, negative sublethal effects on nontarget organisms have been demonstrated for many neonicotinoids (Brandt et al., 2016; Di Prisco et al., 2013; Strobl et al., 2021; Tappert et al., 2017). As a consequence, the use of the neonicotinoids imidacloprid, thiamethoxam, and clothianidin was banned in 2018 in the European Union (European Commission, 2013). However, these neonicotinoids continue to be used in many countries outside Europe (Anand et al., 2021; Mahai et al., 2021), and some members of this class of insecticides such as acetamiprid are still registered in the European Union (Jerez et al., 2022). After the ban of the aforementioned neonicotinoids in the European Union, newly

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developed insecticides such as flupyradifurone and sulfoxaflor are increasingly used by industrial agriculture. These compounds have a similar mechanism of action as the neonicotinoids by inhibiting the synaptic function of neurons as agonists of nicotinergic acetylcholine receptors (nAChRs) and trigger their uncontrolled continuous stimulation (Nauen et al., 2015; Watson et al., 2011). The compounds have a higher affinity for the nAChR in arthropods than for those of mammals, lowering their health risk for humans (Nauen et al., 2015; Watson et al., 2011; Yamada et al., 1999). However, recent studies on bees suggest that flupyradifurone (Siviter & Muth, 2022; Tan et al., 2017; Tosi & Nieh, 2019; Tosi et al., 2021) and sulfoxaflor (Cartereau et al., 2022; Siviter et al., 2018) may also cause negative sublethal effects, such as compromising the learning ability and performance of these pollinators, whereas other studies did not find any adverse effects of sulfoxaflor (Siviter et al., 2019; Tamburini et al., 2021). Another class of insecticides also targeting cholinergic neurons is the organophosphates. A member of this class is the dithiophosphate derivative dimethoate. This compound, while no longer approved in the European Union, is still in use in the United States and in many developing markets. Dimethoate, unlike neonicotinoids, flupyradifurone, and sulfoxaflor, is not an nAChR agonist but inhibits the enzyme acetylcholinesterase.

Sublethal effects of insecticides on beneficial nontarget organisms such as pollinators, predators, and parasitic wasps have been intensively studied for >40 years (reviewed by Desneux et al., 2007; Haynes, 1988). In past years, however, the focus of this research has been on neonicotinoids and bees. Parasitic wasps develop in or on different stages of other arthropods and therefore play an important role as natural enemies maintaining ecological balance (Wang et al., 2019). Bred and released en masse, they can be used for biological pest control (Harush et al., 2021; Waage & Hassell, 1982; Wang et al., 2019). Given that parasitic wasps spend parts of their life cycle feeding on their hosts, including many pest species (Mackauer et al., 1997), they are exposed to the same pesticides as their hosts but have various additional avenues for uptake. Parasitic wasps use floral and extrafloral nectar as well as honeydew as a source of carbohydrates (Dulaurent et al., 2011; Jervis et al., 1993; Lee et al., 2006; Wackers et al., 2008; Wanner et al., 2006) and thus have similar exposure pathways as pollinators. In addition, some parasitic wasps have been shown to consume guttation water (Urbaneja-Bernat et al., 2020) that can contain a considerable amount of pesticides (Hrynko et al., 2021; Reetz et al., 2016; Schmolke et al., 2018). Finally, bioactive amounts of pesticides can be taken up by parasitic wasps simply by tarsal contact with treated plants (Prabhaker et al., 2011). Therefore, it is not surprising that parasitic wasps are also affected by sublethal doses of insecticides (Cook et al., 2016; Desneux et al., 2007; Kang et al., 2018; Kremer & King, 2019; Pisa et al., 2015; Tappert et al., 2017; Teder & Knapp, 2019).

Like most insects, the olfactory sense of parasitic wasps aids them in orienting themselves in complex environments and locating both food resources and hosts (Wang et al., 2003; Zhang et al., 1998). In addition, olfaction plays a major role in pheromone-mediated mate finding, in identifying a mate at close range, and during courtship (Mair & Ruther, 2019; Ruther, 2013). Given that nAChRs are involved in the processing of olfactory stimuli (Dupuis et al., 2012; Rabhi et al., 2016), it is reasonable to assume that sublethal doses of insecticides targeting cholinergic neurons also affect the sense of smell in insects. Consistent with this hypothesis, insecticides targeting nAChRs have been shown to interfere with chemical orientation of parasitic wasps. Males of the egg parasitoid *Trichogramma brassicae* exposed to the organophosphate chlorpyriphos were less arrested by the female sex pheromone (Delpuech, Froment, et al., 1998; Delpuech, Gareau, et al., 1998). Females of the ichneumonid wasp *Microplitis croceipes* feeding on extrafloral nectar contaminated with imidacloprid were impaired in responding to herbivore-induced volatiles released from infested host plants (Stapel et al., 2000). Imidacloprid has also been shown to disrupt olfactory host and mate finding in *Nasonia vitripennis*, a frequently used model organism for the study of parasitic wasp biology (Tappert et al., 2017). Females surviving insecticide treatment no longer responded to the male sex pheromone, and both sexes exhibited altered courtship behavior, resulting in decreased mating rate. In addition, treated females were less successful in olfactory host finding. It is unknown, however, whether other insecticides targeting the cholinergic system of insects have similar effects in *N. vitripennis*.

*Nasonia vitripennis* is a cosmopolitan parasitoid that parasitizes pupae of numerous cyclorraphous flies (Whiting, 1967). Host species of *N. vitripennis* include many flesh flies and blowflies, which not only feed on carrion and other decaying materials but also are pollinators in the adult stage and therefore integral to farmland ecosystems (Currah & Ockendon, 1983; Rader et al., 2009, 2013). Given the ecology of their hosts living adjacent to farmland ecosystems and the problem of pesticides leaching into the wider environment, *N. vitripennis* as a parasitoid is at risk of exposure to sublethal doses of pesticides in agricultural ecosystems and serves not only as a model organism but as a real example of an affected species (Garrido-Bautista et al., 2020). Mate finding in *N. vitripennis* is mediated by a male-derived, substrate-borne sex pheromone that attracts females as long as they are unmated (Lenschow et al., 2018; Ruther et al., 2010). The pheromone is synthesized in the rectal vesicle of males (Abdel-Latief et al., 2008) and consists of (4*R*,5*S*)- and (4*R*,5*R*)-5-hydroxy-4-decanolide and the synergistic minor component 4-methylquinazoline (Ruther et al., 2007, 2008). Close-range mate recognition is mediated by female-derived cuticular hydrocarbons that trigger stereotypic male courtship behavior (Steiner et al., 2006). During courtship, males elicit female receptivity by using an oral aphrodisiac pheromone of unknown chemical structure (van den Assem et al., 1980). After mating, females search for suitable hosts to lay their eggs. They find these hosts through olfactory stimuli, which the hosts passively emit (Frederickx et al., 2014; Steiner & Ruther, 2009).

In the present study, we investigated the sublethal effects of acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor on pheromone-mediated sexual communication and olfactory

host finding in *N. vitripennis*. We applied the insecticides topically to the wasps and performed bioassays to test the response of females to the synthetic male sex pheromone and host odor, respectively. We also examined the mating rates of insecticide-treated couples. Finally, we quantified the amount of a sugar solution ingested by *N. vitripennis* females to discuss bioassay results in the context of insecticide concentrations reported in contaminated nectar.

## MATERIALS AND METHODS

In Germany, experiments with insects do not require Institution Animal Care and Use Committee approval.

### Insects

Wasps of the species *N. vitripennis* were of the strain Phero1 and were originally collected from a bird's nest near Hamburg, Germany (Steiner et al., 2006). Wasps were reared on freeze-killed pupae of the fly species *Lucilia caesar* that were obtained as larvae from a commercial supplier (b.t.b.e. Insektenzucht). Two days after pupation, fly pupae were frozen at −20 °C and used when needed. On Monday, Wednesday, and Friday, wasps of the previous generation of *N. vitripennis* were transposed onto new hosts to copulate and lay eggs for the next generation. Before use, hosts were thawed and dried for at least 2 h in a drying cabinet at 30 °C to prevent the formation of mold.

To make sure that the wasps used in the experiments were virgin and naive, parasitized hosts were dissected and parasitoid pupae removed from their hosts 24–48 h before their estimated emergence dates (generation time at 25 °C and 50% relative humidity is 14–15 days). Fully melanized wasp pupae were isolated from the hosts and transferred singly to 1.5-mL Eppendorf microcentrifuge tube. Tubes were monitored every morning for the appearance of newly emerged wasps. These

wasps were defined as being 0 days old. Wasps were used for the experiment at an age of 1 to 2 days.

### Insecticides

Analytical standards of the tested insecticides, acetamiprid (≥98.0% purity), dimethoate (≥98.0% purity), and flupyradifurone (≥98.0% purity), were sourced from Sigma-Aldrich. All tested pesticides were of PESTANAL®-grade purity. Sulfoxaflor (99.23% purity) was obtained from Dr. Ehrenstorfer. Insecticides were dissolved in acetone (ROTISOLV®, ≥99.8% purity), purchased from Carl Roth.

### Toxicity tests

To have the best possible control over the insecticide dose taken up by the tiny insects, we did not feed the wasps with the active substances but chose topical application of acetone solutions (Tappert et al., 2017). Pure acetone was applied as control, which had no negative effects on the studied parameters in a closely related species (Jatsch & Ruther, 2021). A microinjector (Nanoliter 2010; World Precision Instruments) was used to apply 210 nL of different dilutions (Supporting Information, Table S1) made from 1-mg/mL stock solutions to the abdominal tips of ice-cooled wasps. Groups of eight wasps ($n = 3$ replicates/dose/sex for acetamiprid and dimethoate, $n = 4$ replicates/dose/sex for flupyradifurone and sulfoxaflor) were treated with the insecticides or with pure acetone (control). Mortality was assessed 72 h after application, and dose–mortality curves were generated for each insecticide using the Quest Graph™ LD50 Calculator (AAT Bioquest, 2023). The functional equations of the resulting sigmoidal curves (Figure 1) were used to calculate the doses at which 50% of the treated wasps died (i.e., median lethal dose [LD50]). For the bioassays, three or four doses were chosen for each insecticide that caused mortalities ≤30% in our toxicity



$$Y = 0 + \frac{1-0}{1+\left(\frac{X}{12838.272}\right)^{0.8049}} \qquad Y = 0 + \frac{1-0}{1+\left(\frac{X}{2220.5662}\right)^{1.4341}} \qquad Y = 0 + \frac{1-0}{1+\left(\frac{X}{102248.6228}\right)^{0.8549}} \qquad Y = 0 + \frac{1-0}{1+\left(\frac{X}{14895.9023}\right)^{1.0359}}$$
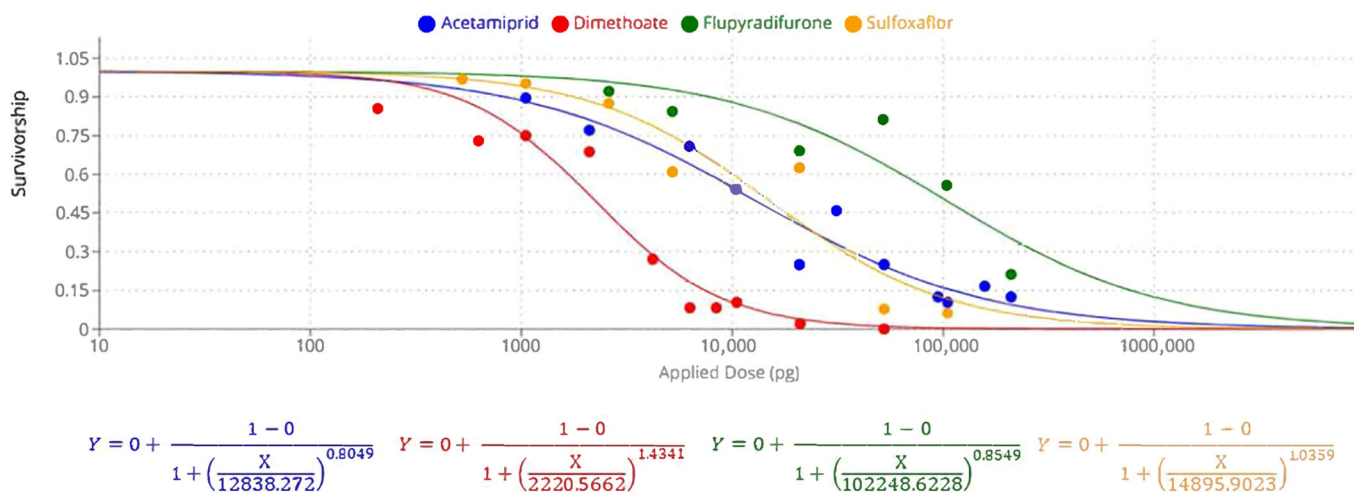
**FIGURE 1:** Dose–mortality curves after 72 h of *Nasonia vitripennis* treated topically with different doses of acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor dissolved in acetone. Dots represent the mean of six groups (three of either sex, acetamiprid, and dimethoate) or eight groups (four of either sex, flupyradifurone and sulfoxaflor) of eight wasps each.

**TABLE 1:** Median lethal dose (LD50) values (evaluated after 72 h) and calculated from the functions of the respective sigmoidal curves using the online tool Quest Graph LD50 Calculator and doses of the four insecticides tested in the bioassays with *Nasonia vitripennis*

| | LD50 (ng/wasp) | Tested doses (ng/wasp) | | | |
|---|---|---|---|---|---|
| Acetamiprid | 12.6 | 0 (96%) | 0.63[a] (n.t.) | 1.05[a] (90%) | 2.1 (77%) | 6.3 (71%) |
| Dimethoate | 2.2 | 0 (94%) | 0.105[a] (n.t.) | 0.21[a] (85%) | 0.63 (73%) | 1.05 (75%) |
| Flupyradifurone | 102 | 0 (98%) | 2.63[a] (92%) | 5.25 (84%) | 21 (70%) | – |
| Sulfoxaflor | 14.9 | 0 (100%) | 0.525[a] (97%) | 1.05[a] (95%) | 2.63 (88%) | 5.25 (61%) |

[a]Sublethal doses, that is, those below the no-observed-effect level (see Supporting Information, Table S1).
Values in parentheses represent the percentages of surviving wasps in the toxicity tests.
n.t. = doses were tested in the bioassays but not in the toxicity tests.

tests. As for sulfoxaflor, one additional dose was tested that caused a higher mortality (39%; Table 1).

## Effects on pheromone communication

The effect of insecticides on the pheromone response of *N. vitripennis* females was tested by isolating and treating virgin female *N. vitripennis* with different doses of the four insecticides or acetone (control; $n = 20$ per treatment); 24 h after the treatment, females were tested in a dual-choice olfactometer, as described in previous studies (Ruther et al., 2014; Tappert et al., 2017). Briefly, 1 µL of the synthetic sex pheromone dissolved in dichloromethane (200 ng/µL (4$R$,5$S$)-5-hydroxy-4-decanolide, 100 ng/µL (4$R$,5$R$)-5-hydroxy-4-decanolide, and 3 ng 4-methylquinazoline, synthesized as described previously (Ruther et al., 2007, 2008, 2016), was applied to a disk of filter paper. Control paper disks were treated with the same amount of pure dichloromethane. After evaporation of the solvent, test and control disks were put into the test and control cavity of the olfactometer, and treated females were released individually into its center. The time females spent in either cavity of the olfactometer was recorded using The Observer XT 15 software (Noldus Information Technology). The olfactometer was rotated 90° after every observation to avoid bias due to external influences.

## Effects on mating rate

To test the influence of the four insecticides on mating rate, virgin male and female *N. vitripennis* were isolated and separated into three groups ($n = 20$/dose/group), depending on whether the male, the female, or both partners had been treated with insecticide doses or pure acetone (control), respectively. Couples of treated wasps were then placed in a 2-cm-diameter plexiglass arena and observed for 5 min using a stereomicroscope. Successful copulations during the observation time were recorded and converted into a percentage value for each dose and group constellation for further analysis.

## Effects on host finding

To test whether the four insecticides influence the ability of females to locate hosts by olfactory cues, newly emerged females (<1 day old) were mated and subsequently treated with an insecticide dose or the pure solvent ($n = 20$/dose/treatment). Treated females were held for 24 h and then tested in a T-olfactometer (Figure 2). This olfactometer was divided into four zones: (a) the start zone, where the microcentrifuge tube with the female was attached at the beginning of each observation; (b) the neutral zone, which included a corridor to the tested zones and a buffer between them; (c) the control zone, to which an empty microcentrifuge tube was connected; and (d) the host zone, on the opposite side of the control zone, to which a microcentrifuge tube with five *Lucilia caesar* pupae was connected. Polyamide gauze (mesh width 125 µm) was installed between microcentrifuge tubes of test and control zones, allowing host volatiles to diffuse into the olfactometer but excluding visual cues. Females were observed for 5 min after the microcentrifuge tube with the female had been attached to the olfactometer. The time the females spent in test and control zone was recorded using The Observer XT 15 software.

## Quantification of the amount of artificial nectar consumed by *N. vitripennis*

**Feeding experiment.** A likely major source through which parasitic wasps ingest insecticides is the consumption of contaminated nectar. To obtain a realistic basis for discussion of the ecological relevance of the bioassay results, the amount of an artificial nectar (30% glucose solution in water) consumed by *N. vitripennis* females within 48 h was quantified by gas chromatography/mass spectrometry (GC/MS). Samples (10 µL each) of a 30% (m/v) solution of α-D-glucose (Sigma-Aldrich) were pipetted into 1.5-mL microcentrifuge tubes, and one *N. vitripennis* female per tube was added ($n = 15$). Females were allowed to feed on the artificial nectar for 48 h. To ensure that the wasps had expended some of their energy resources and to increase their motivation to feed, females were mated before the experiment and given the opportunity to lay eggs for 2 days. Lids of the tubes were perforated with a needle for aeration. Control tubes ($n = 15$) were prepared the same way without adding a wasp.

**Sample preparation.** After 48 h, wasps were removed, and the residues of the glucose solution were redissolved in 20 µL of water containing 3 mg of D-(+)-mannose as an internal standard. The sugar solutions were transferred to 1.5-mL GC vials, and
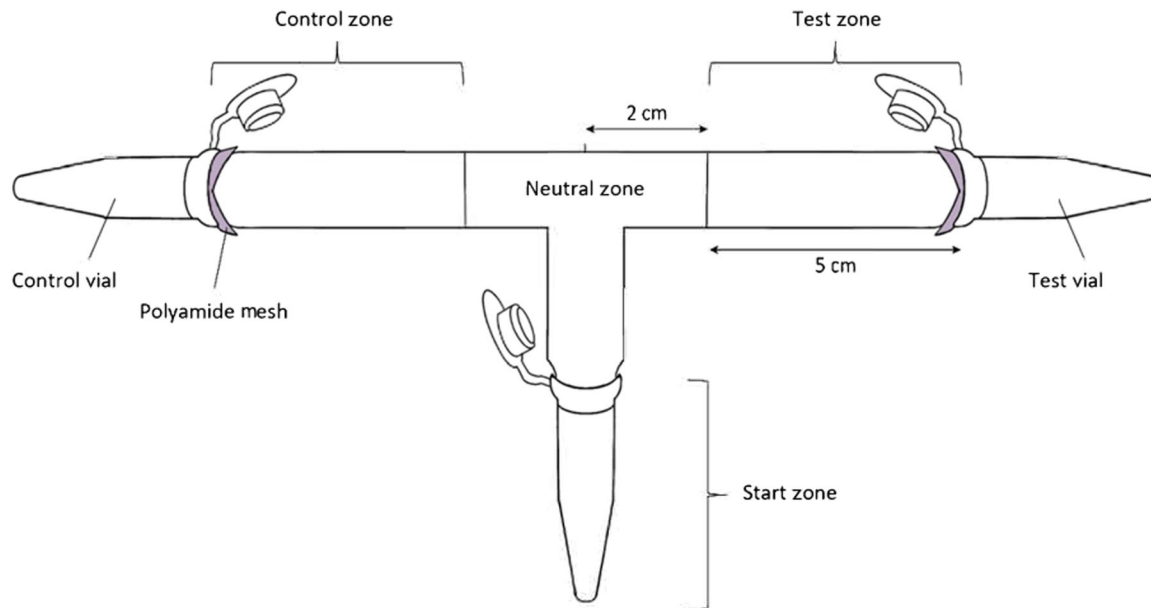
**FIGURE 2:** Schematic of the T-olfactometer used in the host-finding experiments (for details, see text).

microcentrifuge tubes were rinsed with another 20 µL of distilled water which were added to the respective samples. Sugar solutions were lyophilized overnight, and sugar residues were derivatized for GC/MS analysis by a two-step derivatization method (Yi et al., 2014). In the first step, sugars were oximized by adding 200 µL of a solution of methoxamine hydrochloride (5% dissolved in pyridine; Sigma-Aldrich) and incubating the vials for 30 min at 75 °C. This step reduced the number of possible sugar isomers to two per sugar (*cis*- and *trans*-oximes), facilitating the analysis by GC/MS. In the second step, 50 µL of each sample were transferred to new GC vials, and the oximes were silylated by adding 40 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich) and incubating the vials for another 30 min at 75 °C. The vials were allowed to cool, 900 µL of dichloromethane were added, and the samples were diluted 1 to 5 with the same solvent prior to GC/MS analysis.

**GC/MS analysis.** The GC/MS analysis was performed on a Shimadzu QP2010 GC/MS system operated in electron ionization mode at 70 eV and equipped with a 60-m BPX-5 capillary column (inner diameter 0.25 mm, film thickness 0.25 µm). Helium was used as carrier gas at a linear velocity of 40 cm/s. Samples were injected in split mode (1:25) at 300 °C using an AOC20i autosampler. The temperature program started at 70 °C, was increased at 20 °C/min to 160 °C, then at 4 °C/min to 180 °C, and finally at 10 °C/min to 300 °C (held for 10 min). Glucose amounts were determined by relating the total peak area of the two glucose peaks to the total peak area of the two mannose peaks (internal standard).

## Statistical analyses

All statistical analyses were performed using R (Ver. 4.0.1; 2023). If data did not meet the assumptions for parametric testing, nonparametric tests were used. Survival rates of insecticide-treated wasps were compared with the respective controls using Fisher's exact test. Pheromone and host finding data were analyzed within each dose using a Wilcoxon matched-pairs test. Copulation rates of insecticide-treated and control couples were analyzed across all doses using a Fisher's exact test. If this test showed a significant result ($p < 0.05$), pairwise comparisons were done between the control dose and each insecticide dose. The glucose residues in the centrifuge tubes of the fed females and the control group were each averaged and analyzed using a *t* test. Box plots and bar plots in the results were plotted using the PAST 4.03 software (Hammer et al., 2001).

## RESULTS

### Toxicity tests

The dose–mortality curves revealed clear differences in the acute toxicity (Figure 1 and Table 1) of the four insecticides. Dimethoate showed the highest toxicity (LD50 = 2.2 ng/wasp), while flupyradifurone was the least toxic of the four (LD50 = 102 ng/wasp). Acetamiprid (LD50 = 12.6 ng/wasp) and sulfoxaflor (LD50 = 14.9 ng/wasp) had similar, intermediate toxicities. A sublethal dose is defined as a dose inducing no statistically significant mortality in the experimental population (Desneux et al., 2007). Accordingly, the no-observed-effect levels (NOELs) for acetamiprid, dimethoate, flupyradifurone, and sulfoxaflor were determined as 1.05, 0.21, 2.63, and 1.05 ng, respectively (Table 1; Supporting Information, Table S1). Doses below these NOELs were considered sublethal.

### Effects on pheromone communication

Virgin control females treated with pure acetone spent significantly more time in the pheromone cavity than in the untreated control cavity in all experiments (Figure 3A–D). In contrast, females treated with any dose of acetamiprid or
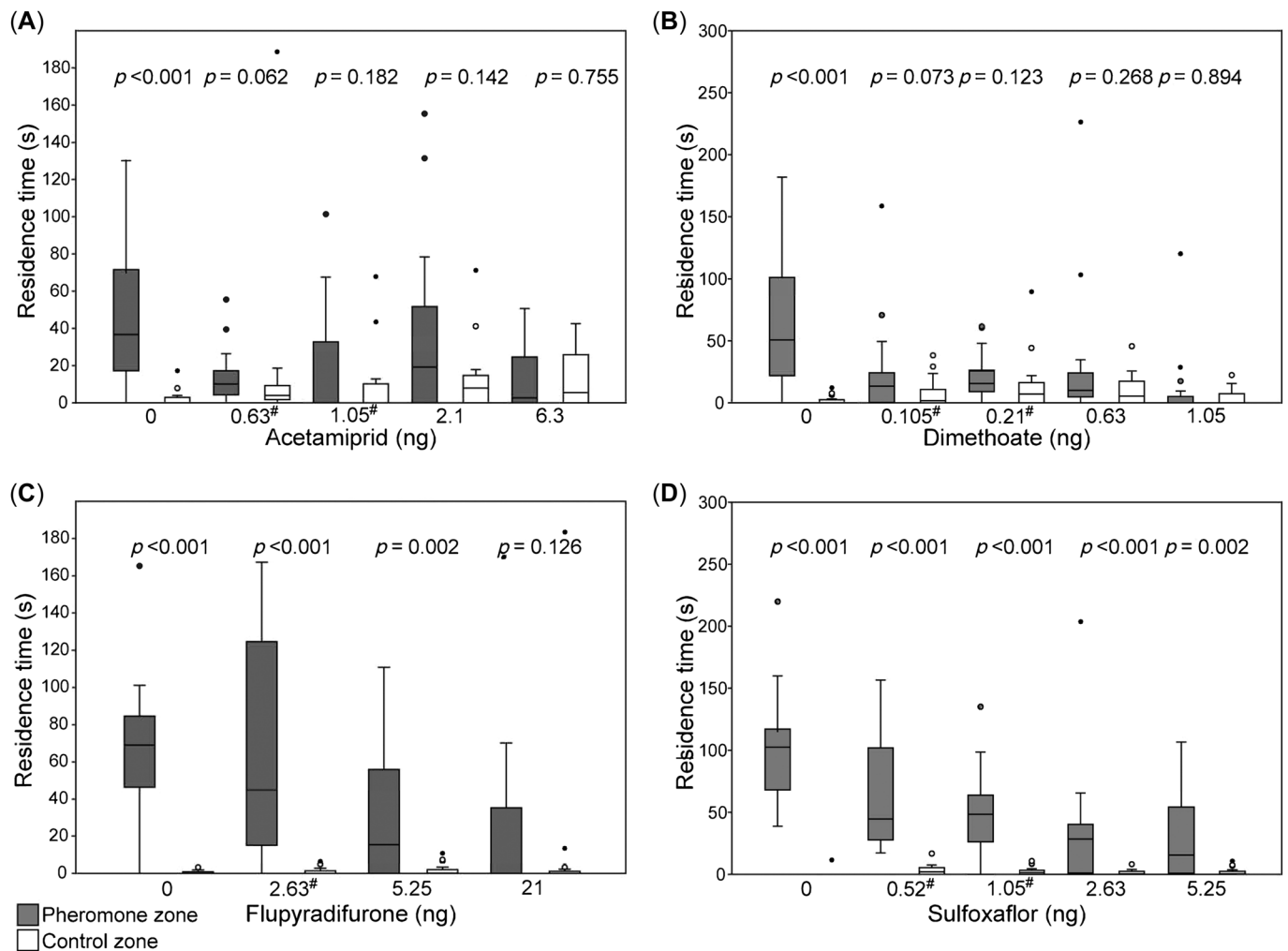
**FIGURE 3:** Effect of different doses of the four insecticides on the response of virgin *Nasonia vitripennis* females to the synthetic male sex pheromone. Females were treated with either pure acetone (0 ng, control) or doses of (**A**) acetamiprid, (**B**) dimethoate, (**C**) flupyradifurone, or (**D**) sulfoxaflor. #Sublethal doses. One day after application the females were tested in a two-choice olfactometer. Shown are the residence times of females in the pheromone cavity and the solvent-treated control cavity of the olfactometer. Box-and-whisker plots show median (horizontal line), 25% to 75% quartiles (box), maximum/minimum range (whiskers), and outliers (° means >1.5 × and * means >3 × box height). Statistical analysis for each treatment by Wilcoxon matched pairs test ($n = 20$).

dimethoate no longer preferred the pheromone cavity (Figure 3A,B). Both insecticides, however, showed a relatively high degree of variability, as depicted by numerous outliers. Flupyradifurone disrupted the pheromone preference of virgin females only at the highest tested dose (Figure 3C), whereas sulfoxaflor doses had no significant effect on the pheromone response of virgin females at tested levels (Figure 3D).

## Effects on the mating rate

Tested doses of any of the four insecticides reduced the mating rate of treated couples significantly in at least one of the tested concentrations/constellations (Figure 4A–D). When only males were treated, all four substances significantly reduced the mating rate of couples at the highest tested doses (Figure 4A–D). When only the females were treated, only flupyradifurone and sulfoxaflor had significant effects at the highest tested dose (Figure 4C,D). When both partners

were treated, acetamiprid and sulfoxaflor decreased mating rates of treated couples significantly at the highest tested doses (Figure 4A,C). Flupyradifurone caused significant effects at any of the tested doses (Figure 4C), whereas dimethoate had no significant effects at the tested dose range (Figure 4B).

## Effects on host finding

In all experiments, mated females treated with pure acetone spent significantly more time in the host zone of the T-olfactometer than in the untreated control zone (Figure 5A–D). This preference for host odor, however, was absent in females treated with any dose of the four insecticides except for the lowest doses of acetamiprid and flupyradifurone, respectively. Remarkably, females treated with doses ≥0.21 ng of dimethoate even avoided the host zone significantly (Figure 5B).
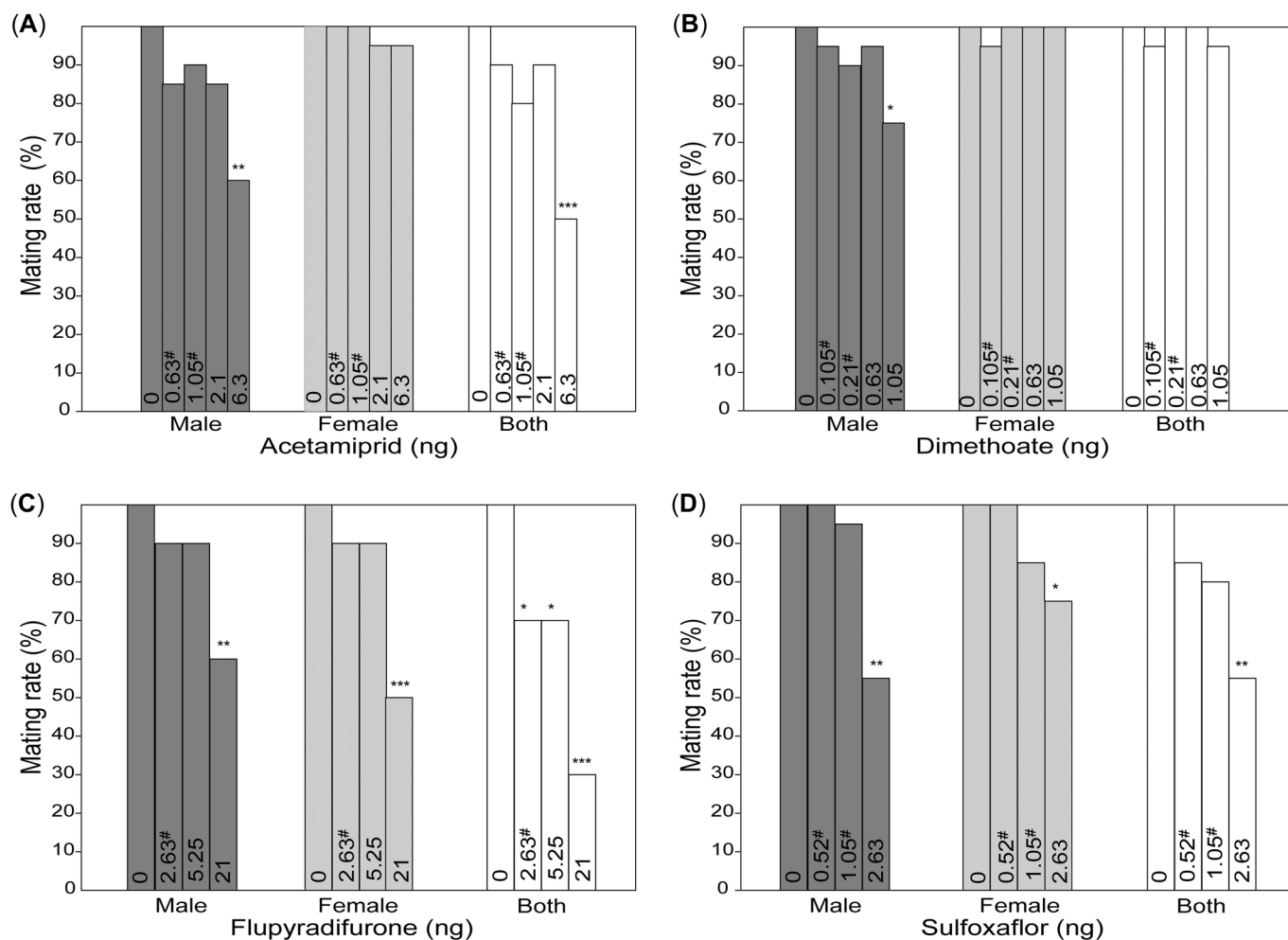
**FIGURE 4:** Effect of different doses of the four insecticides on the mating rates of virgin *Nasonia vitripennis*. Shown are the mating rates of *N. vitripennis* couples, where either the male partner, the female partner, or both partners were treated with either pure acetone (0, control) or a dose (doses in nanograms given in the bars) of (**A**) acetamiprid, (**B**) dimethoate, (**C**) flupyradifurone, or (**D**) sulfoxaflor. #Sublethal doses. Significant differences between the individual sublethal doses and the control are marked by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 20$, Fisher's exact test).

## Quantification of the amount of artificial nectar consumed by *N. vitripennis*

Mean glucose residues in the microcentrifuge tubes after 48 h feeding by *N. vitripennis* females were significantly lower (mean ± SE 1.94 ± 0.11 mg) than in the control tubes (2.37 ± 0.09 mg; *t* test, $p = 0.0048$). Thus, female wasps consumed 18% of the total amount supplied to them within 48 h, which corresponds to 0.43 mg. Considering the sample volume of 10 µL applied in the feeding experiment, females consumed approximately 1.8 µL of the artificial nectar.

## DISCUSSION

In the present study, we extend our knowledge of the adverse effects of pesticides on beneficial nontarget organisms by showing that sublethal doses of four insecticides targeting the insect cholinergic system impair important traits related to olfaction in the parasitic wasp *N. vitripennis*. Thus, not only imidacloprid (Tappert et al., 2017; Whitehorn et al., 2015), one of the neonicotinoids banned in the European Union, but also the still-approved neonicotinoid acetamiprid and the next-generation nAChR agonists flupyradifurone and sulfoxaflor, as well as the acetylcholinesterase inhibitor dimethoate, interfere with the chemical sense-mediated performance of this model organism. Mate and host finding are crucial prerequisites for the reproductive success of parasitic wasps. Females that are unable to locate their hosts for oviposition are also unable to produce any offspring. Those that remain unmated, even if they could find hosts, are forced to produce all-male offspring as a result of haplodiploid sex determination in hymenopterans (Beukeboom & van de Zande, 2010). Given that *N. vitripennis* and many other parasitoids prefer to produce female-biased offspring because of local mate competition (Quicke, 1997), female virginity is another fitness-relevant factor in these species that might be caused by sublethal insecticide doses. Therefore, our results suggest that doses of the four insecticides, at which the vast majority of the exposed wasps survive, compromise the important ecosystem function these insects have as natural enemies as well as their applicability in
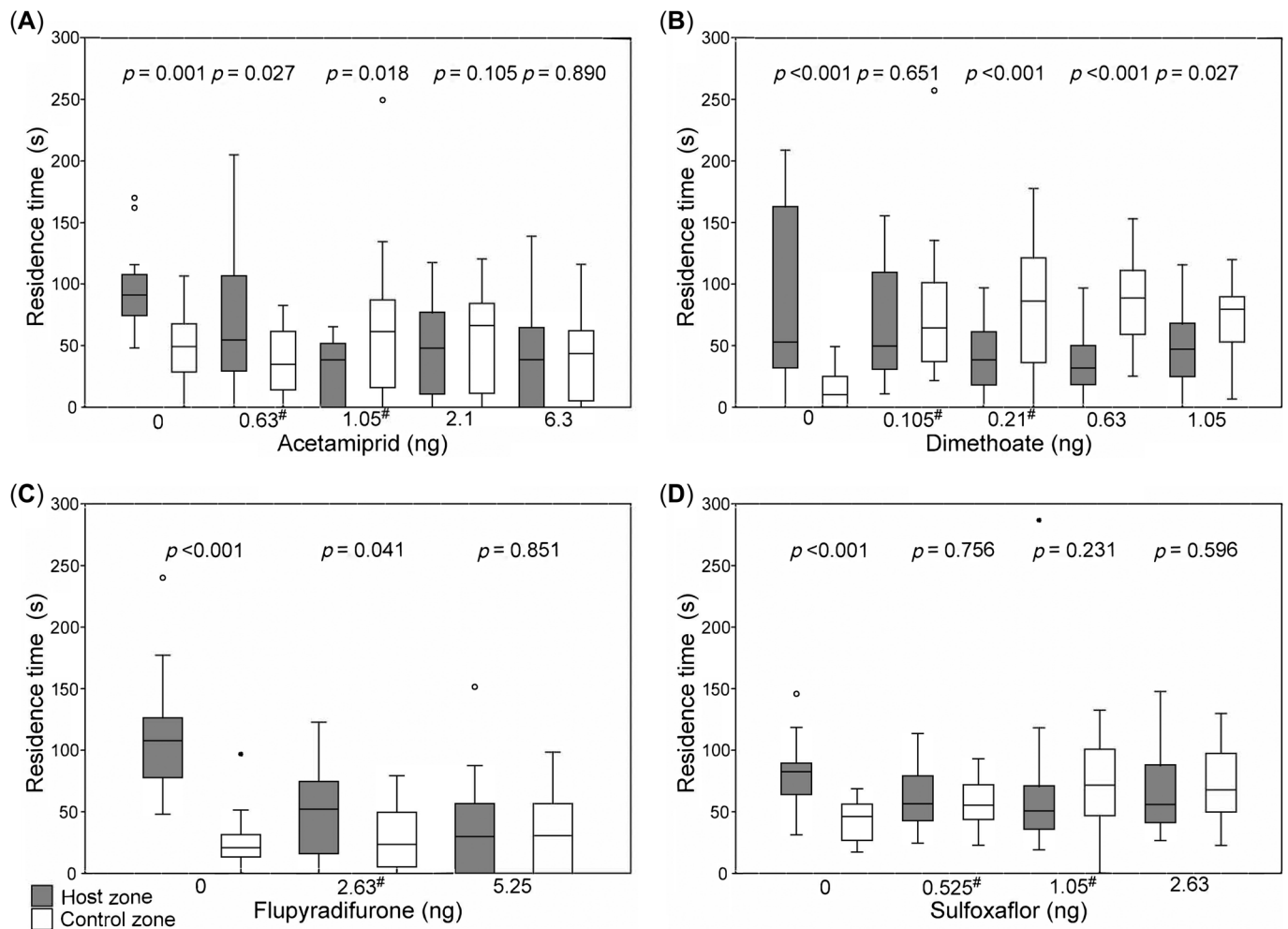
**FIGURE 5:** Effect of different doses of the four insecticides on the response of mated female *Nasonia vitripennis* to host odor (pupae of *Lucilia caesar*). Mated females were treated with pure acetone (0, control) or a dose of (**A**) acetamiprid, (**B**) dimethoate, (**C**) flupyradifurone, or (**D**) sulfoxaflor. #Sublethal doses. One day after application, females were tested in a two-choice T-olfactometer. Shown are the residence times of females in the host zone and the untreated control zone of the olfactometer. Box-and-whisker plots show median (horizontal line), 25% to 75% quartiles (box), maximum/minimum range (whiskers), and outliers (° means >1.5 × and * means >3 × box height). Statistical analysis for each treatment by Wilcoxon matched pairs test ($n = 20$).

the context of biocontrol. Although this suggestion is supported by previous studies that have also found sublethal effects of these compounds in parasitic wasps (Costa et al., 2023; Gao et al., 2023; Mohammed & Karut, 2021), further experiments under more realistic semifield conditions are needed for a definitive evaluation.

The sublethal effects of the four insecticides varied with the ecological context in which they were investigated and depended on the dose tested, with the lowest bioactive doses correlating largely with the acute toxicity (LD50) of the respective compounds. Dimethoate had the highest acute toxicity for *N. vitripennis* and disrupted pheromone communication and host finding even at doses as low as 0.1 ng/wasp. The mating frequency was only affected at a 10-fold higher dose (1.05 ng/wasp) of dimethoate when applied to males. However, this result could have occurred by chance because the effect was weak (Figure 4B) and no longer present when both males and females were treated. Flupyradifurone exhibited the lowest acute toxicity, and consequently, significantly higher

doses were necessary to influence both the pheromone response (21 ng) and olfactory host finding (5.25 ng), while it affected mating rate at a sublethal dose of 2.63 ng, which was comparable with the other agents. Acetamiprid impaired the pheromone response of *N. vitripennis* females at subnanogram levels, while nanogram doses were necessary to affect the mating rate and to disrupt olfactory host finding. Sulfoxaflor had no effects on the pheromone response at the tested dose range but affected olfactory host finding even at a sublethal dose of 0.525 ng. This suggests that pheromones and host volatiles are processed differently and that behavioral reactions are differentially influenced by insecticide residues in *N. vitripennis* females. This idea is supported by the fact that dimethoate disrupted the pheromone response, while the preference for host odor even turned into avoidance at doses ≥0.21 ng. Interestingly, chlorpyrifos, another organophosphate insecticide, disrupted the male pheromone response in the egg parasitoid *T. brassicae* (Delpuech, Froment, et al., 1998; Delpuech, Gareau, et al., 1998), while it even increased the response of

females to host-associated kairomones in the *Drosophila*-parasitoids *Leptopilina heterotoma* and *L. boulardi* (Delpuech et al., 2005; Rafalimanana et al., 2002). Pheromones in insects are often perceived and processed by specialized components of the olfactory system (pheromone receptor proteins, pheromone-binding proteins, macroglomerular complex in the antennal lobe) that differ from those for general, food-associated odorants (Renou, 2014). The mechanisms underlying the differing sublethal effects of insecticides on the response to pheromones and host-associated volatiles, respectively, deserve further research in the future. Our results, however, underline that possible sublethal effects on the perception of chemical stimuli need to be investigated in different contexts.

An important question arising from our results is whether the tested insecticide doses are field-realistic and ecologically relevant. Again, further research is needed because the uptake pathways of insecticides in parasitic wasps have not been fully explored. In the present study, we deliberately chose topical application (as an acetone solution) for the administration of the active ingredients because this allows more control of the absorbed doses. Uptake of bioactive doses via the cuticle or tarsi after exposure to insecticide aerosols or contact with treated surfaces is a possible route by which bioactive amounts of insecticides can enter parasitic wasps (Delpuech et al., 2005; Prabhaker et al., 2011; Salerno et al., 2002). Ingestion of insecticides via the consumption of contaminated floral and extrafloral nectar, however, is probably the primary route by which parasitic wasps in natural habitats may be exposed to insecticides (Jervis et al., 1993; Rose et al., 2006; Stapel et al., 2000; Supporting information, Table S2). Our data show that females with partly depleted energy resources consumed approximately 2 µL of an artificial nectar within 48 h. Based on this amount, literature data on nectar contamination with the insecticides we studied (Supporting information, Table S2) indicate that the highest concentrations reported for the four active ingredients are in the range of, or slightly below, concentrations that would cause sublethal effects in *N. vitripennis*. In addition, it must be considered that some active substances have a stronger effect when taken up orally than when applied topically (Tosi et al., 2022) and that even relatively closely related species may exhibit drastic differences in their susceptibility to insecticides (Hayward et al., 2019). Hence, other parasitic wasps may be impaired in their olfactory capabilities by even lower doses of the insecticides. Furthermore, at least neonicotinoids can have cumulative effects (Huang et al., 2021; Sanchez-Bayo & Tennekes, 2020; van der Sluijs et al., 2013). Thus, multiple ingestions of very low doses could ultimately lead to measurable sublethal effects. Insecticides are often used in multicomponent blends (Yu & Ting, 2019), and the simultaneous use of pesticides from other classes (fungicides, herbicides; Schuhmann et al., 2022) may result in exposure of parasitic wasps to multiple active substances, which may cause additive or synergistic adverse effects (Tosi et al., 2022; Wang et al., 2020; Willow et al., 2019). Guttation water from plants grown from coated seeds may contain significant amounts of insecticides (e.g., up to ~100 ng/µL thiacloprid, 150 ng/µL

thiamethoxam, and up to 200 ng/µL imidacloprid; Girolami et al., 2009), although no literature data are available for the insecticides tested in the present study. It has been shown, however, that parasitic wasps use guttation water as a food source (Urbaneja-Bernat et al., 2020) and that guttation water from arable wild weeds may also contain systemic pesticides in significant amounts if they grow near treated crops (Mörtl et al., 2019). Honeydew, the sugary excretion of aphids and other Hemiptera, is also commonly used by parasitic wasps as a carbohydrate source and may contain biologically active levels of insecticides (Calvo-Agudo et al., 2019, 2022). Because all nAChR agonists tested in the present study are widely used against hemipteran pests such as aphids, whiteflies, and thrips (Elbert et al., 2008; Nauen et al., 2015; Zhu et al., 2011), it can be expected that these substances may enter beneficial insects also via honeydew, although data on residue levels are not yet available. The question of whether parasitic wasps, because of their particular lifestyle, can also take up bioactive doses of pesticides when developing in or host-feeding on contaminated hosts and whether the ingested amounts are sufficient to affect olfactory responses is still largely unresolved and requires further research. This aspect could become an increasing problem if parasitic wasps parasitize resistant hosts that are able to survive despite insecticide ingestion (Umoru & Powell, 2002).

Many features of the olfactory system are largely conserved in insects (Hansson & Stensmyr, 2011), and therefore it is unlikely that the sublethal effects demonstrated in the present study are unique for *N. vitripennis* or parasitic wasps, respectively. Because of the special importance of olfaction to insect performance, additional species from other insect guilds need to be studied to determine whether insecticides targeting nAChRs also affect the mating and foraging behavior of these species and in this way contribute, largely unnoticed, to the current decline of many insect species. Our study supports the claim that sublethal effects should be given more consideration in the approval process of new pesticides (Siviter et al., 2023). Because of the ease of breeding, the availability of well-established and easily performed bioassays, and the extensive knowledge available on chemical communication (Mair & Ruther, 2019), the *Nasonia* model system offers excellent conditions for future use in such registration procedures.

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