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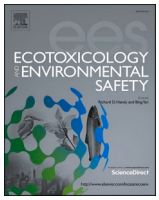

Multifaceted sublethal effects of neurotoxic insecticides on olfactory-mediated orientation in the spotted wing drosophila

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ABSTRACT

Neurotoxic insecticides are recognized and classified by their primary mode of action, but they frequently exhibit secondary targets and downstream consequences that are often neglected. Hazards to arthropod physiology and behavior can sublethally occur through environmentally relevant pesticide residues and/or by contact with non-targeted developmental stages. *Drosophila suzukii* is a key invasive pest of soft-skinned fruits and provides a suitable model to study how neurotoxicants interfere with olfactory-mediated orientation, a key process underlying host detection and oviposition. However, information on how neurotoxic insecticides influence olfactory-driven orientation behavior in this pest remains undocumented. This study investigated the sublethal effects of neurotoxic insecticides (chlorpyrifos, cyazypyr (cyantraniliprole), lambda-cyhalothrin and spinosad) at realistic occurrence ranges (i.e., estimated lethal concentrations 10%, LC_{10}) on adult female orientation. Contamination occurred either by residual contact at the adult stage or ingestion during larval development. Behavioral responses were assessed using a Y-tube olfactometer, while electroantennography (EAG) and quantitative expression of nine olfactory receptor (*Or*) genes evaluated peripheral sensorial capacity. Under adult residual contact exposure, chlorpyrifos and spinosad disrupted olfactory orientation in adult flies. In addition, these insecticides, together with lambda-cyhalothrin, increased decision-making time. Residual exposure reduced antennal sensitivity and downregulated *Or* gene expression. In contrast, following larval ingestion, all tested insecticides induced adult behavior disorientation without detectable effects on peripheral olfactory function. Overall, these findings highlight the importance of considering multiple exposure pathways and sublethal neurotoxic effects when evaluating insecticide hazards on targeted insects, with implications for *D. suzukii* ecology and the efficacy of olfactory-based integrated pest management strategies.

1. Introduction

Olfaction plays a fundamental role in animal behavior and niche specialization, a pattern shaping key processes such as host location and mating across taxa, as clearly observed within insects of the Drosophilidae (Diptera) family (Keesey et al., 2022). Among them, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) is a key pest of soft-skinned fruits and exhibits a pronounced olfactory specialization for ripe and fresh fruits, associated with a distinct repertoire of odorant receptor (*Or*) genes compared with other *Drosophila* species (Keesey et al., 2015; Karageorgi et al., 2017; Ramasamy et al., 2016). Moreover, *D. suzukii* can dynamically modulate its olfactory system according to the physiological state and environmental conditions (Gadenne et al., 2016).

Crava et al. (2019) demonstrated mating-dependent olfaction plasticity in *D. suzukii* females, characterized by differential antennal *Or* expression that matches reproductive needs and drives behavioral preference towards fresh fruits. For these reasons, the olfactory system

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of *D. suzukii* has become important within integrated pest management (IPM) research (Asplen et al., 2015; Tait et al., 2021), stimulating the development of monitoring and behavioral manipulation strategies, such as mass trapping, attract and kill and push and pull techniques (Babu et al., 2022; Gugliuzzo et al., 2024; Wallingford et al., 2018). Nevertheless, the rapid population increase of *D. suzukii* close to fruit harvesting often requires prompt management practices, including insecticide use (Fellin et al., 2024; Tait et al., 2021). Current insecticide application protocols against *D. suzukii* primarily rely on broad-spectrum neurotoxic compounds, including organophosphates, pyrethroids, spinosyns, and diamides (Lisi et al., 2024a; 2024b; Tait et al., 2021).

Once applied, such chemicals persist in the environment while degrading into low and frequently sublethal concentrations, as reported for pyrethroids and systemic insecticides (Desneux et al., 2005; Guedes et al., 2023, 2026). This phenomenon results in altered physiology and behavior in surviving individuals at environmentally relevant conditions (Guedes et al., 2016, 2017; Krüger et al., 2021; Lewandowska-Wosik et al., 2024; Shaw et al., 2019), including impairment of insect sensory systems and olfactory performance, ultimately disrupting intra- and interspecific communication (Desneux et al., 2007; Ricupero et al., 2020; Tricoire-Leignel et al., 2012). Such behavioral alterations may result from reduced antennal sensitivity, altered *Or* gene expression, and disrupted signal transmission from olfactory receptor neurons to higher brain centers, including the mushroom bodies in insects (Tricoire-Leignel et al., 2012). Although sublethal insecticide effects on *D. suzukii* orientation behavior have been reported (Guedes et al., 2018), the underlying impacts on the peripheral olfactory system remain undocumented. Moreover, a critical and often overlooked aspect concerns the potential carry-over effects of pesticide exposure from juvenile to adult stages (Lisi et al., 2023).

Exposure of larvae to neurotoxicants can induce abnormal neuronal development and reduced neurogenesis, with permanent consequences for adult olfactory perception, learning and memory (Ke et al., 2023). This issue can be relevant for *D. suzukii*, as larvae frequently develop on damaged, rotten or overripe fruits that are often contaminated with pesticide residues (Wang et al., 2016; Wise et al., 2015). In this context, two companion studies characterized the concentration-mortality response of *D. suzukii* larvae (Lisi et al., 2023) and adults (Lisi et al., 2024c) exposed to various neurotoxicants by feeding and residual contact, respectively. These studies demonstrated that LC_{10} values (i.e., lethal concentrations causing 10% mortality) were several orders of magnitude lower (from 500- to 10^{13}-fold) than insecticide label concentrations and that sublethal effects included prolonged development time and reduced puparium size following larvae exposure (Lisi et al., 2023), as well as decreased survival and fertility in adults after residual exposure (Lisi et al., 2024c).

Such concentrations are environmentally relevant for the tested insecticides (Lisi et al., 2023, 2024c), as monitoring studies conducted across Europe and South America have reported residues in agricultural environments at concentrations ranging from 1 to 162 μg/kg, with measurable adverse effects on resident biota (Alaoui et al., 2024). Moreover, these LC_{10} values (Lisi et al., 2023, 2024c) did not exceed the maximum residue limits (MRLs) legally permitted in berries under the European Union pesticide regulatory framework, i.e., approximately 4–6 mg/kg for cyazypyr and 0.05–1.5 mg/kg for chlorpyrifos, spinosad, and lambda-cyhalothrin (European Commission, 2005). Therefore, these levels of exposure reflect realistic occurrence ranges and represent conservative, environmentally meaningful scenarios.

This study aimed at investigating the sublethal effects of LC_{10} of four neurotoxic insecticides (chlorpyrifos, cyazypyr, lambda-cyhalothrin and spinosad) on the olfactory-mediated orientation behavior in *D. suzukii*, following either residual exposure of adults or larval ingestion. An integrative approach was employed, combining olfactometer bioassays with electroantennography (EAG) and molecular analyses. By linking exposure route, sensory disruption, and behavioral outcomes, our findings can contribute to a better understanding of the secondary and low-concentration effects of insecticides and their implications for attractant-based IPM strategies targeting *D. suzukii*.

2. Materials and methods

2.1. Biological material

The laboratory colony of *D. suzukii* originated from individuals collected on infested blackberries (*Rubus* sp.) in Eastern Sicily (N 37°39'47.39" - E 14°54'3.06") in September 2015. Adult flies were reared on a standard cornmeal artificial diet (Lisi et al., 2023), provided in rearing tubes (Ø × h: 25 × 95 mm) for both egg-laying and larval development. Adults were maintained inside BugDorm® cages (MegaView, Taiwan, type 32.5 × 32.5 × 32.5 cm) under standardized laboratory conditions (22 ± 2°C, 60 ± 10% R.H., 14 L:10D photoperiod) and supplied *ad libitum* with a honey-water solution (1:1) using a food dispenser.

2.2. Insecticides

Four neurotoxic insecticides with different modes of action (MoA) were selected for this study. All the tested insecticides are widely used against *D. suzukii* or other key pests affecting the same crops and included three synthetic compounds: chlorpyrifos (Dursban®, Dow AgroSciences S.r.l.; IRAC group 1B: acetylcholine esterase inhibitor), cyazypyr (Exirel®2016, FMC Agro Italia; IRAC group 28: ryanodine receptor agonist) and lambda-cyhalothrin (Karate Zeon CC®, Syngenta Italia S.p.a; IRAC group 3 A: sodium channel modulator); and one compound of microbiological origin, spinosad (Laser®, Dow AgroSciences S.r.l.; IRAC group 5: nicotinic acetylcholine receptor/GABA modulator) (Table S1).

Despite chlorpyrifos has been subjected to significant regulatory restrictions in the EU (European Union, 2026), it was included in the study as a broad-spectrum reference insecticide. For each insecticide, the LC_{10} was previously estimated in two companion studies on *D. suzukii* larvae (Lisi et al., 2023) and adults (Lisi et al., 2024c) (Table S1). The LC_{10} solutions were prepared through a series of dilutions starting from a stock insecticide solution at the recommended field dose, according to the manufacturer instructions, under a fume hood (Table S1).

2.3. Residual contact and ingestion exposure

Residual contact exposure of *D. suzukii* adults to insecticide LC_{10} was carried out following Lisi et al. (2024c). Briefly, five adult couples (i.e., five females and five males, three days old) were exposed for 72 h to contaminated strawberry leaves inside a two-cup arena system, i.e. two superposed plastic glasses, see Lisi et al. (2024c). Leaves were previously dipped in each LC_{10} insecticide solution or water-surfactant solution (Tween 80®,10 μL/L of water) for the control and allowed to dry for one hour under the fume hood. Following the 72-hour sublethal exposure, surviving mated *D. suzukii* females were assayed in olfactometer, electroantennography, or transcriptomic analysis. Ingestion exposure of *D. suzukii* larvae to insecticide LC_{10} was performed according to Lisi et al. (2023). Adults were allowed to oviposit on untreated artificial diet and, after 24 h, ten first-instar larvae were gently transferred to rearing tubes containing 5 mL of artificial diet previously contaminated with each LC_{10} insecticide solution. The untreated control consisted of an uncontaminated diet prepared following the same procedure. Tubes were maintained under standard experimental conditions (22 ± 2°C, 60 ± 10% R.H., 14 L:10D photoperiod) until adult emergence.

After emergence, three days old females were individually transferred to a clean rearing tube with an untreated male from the colony for mating for 72 h and provided with drops of honey-water solution (1:1) on the inner surface of the tube lid as a food source. Mated females were

then used for behavioral, physiological, or molecular assays, as described below. For each exposure route, independent biological material was used for olfactometer, electroantennography and transcriptomic analyses.

2.4. Olfactometer bioassays

The effect of insecticide exposure at LC_{10} on the olfactory response of mated *D. suzukii* females following residual and ingestion exposure were evaluated using a dual choice Y-tube olfactometer (Analytical Research Systems, Gainesville, FL). The device consisted of a transparent glass tube (3 cm of internal diameter) with an entry arm 61 cm long and two side arms 44 cm long, forming a 70° inside angle. The arms were connected via plastic PTFE tubing to two 5 L glass jars containing odor sources. One jar delivered clean air as control, while the other contained a beaker with 50 mL of *Droskidrink* (75% apple cider vinegar, 25% red wine, 4 g of brown sugar) and a second beaker containing a solution of 50 mL of water, 10 g of yeast, and 4 g of sugar. These blends are commonly used as attractants for field monitoring of *D. suzukii* (Hamby and Becher, 2016). The Y-tube was connected to an air pump (Airfizz®, Ferplast) generating a unidirectional airflow (150 mL/min) from the arms to the base of the tube. The olfactometer was illuminated with 2000 lux light intensity positioned 80 cm above the arms, simulating twilight conditions corresponding to peak *D. suzukii* activity (Hamby et al., 2016). The olfactometer was positioned horizontally on a white table to minimize visual cues and placed in a dark room at 22 ± 1 °C and 60 ± 10% R.H.

Mated females from residual and ingestion exposures were starved in *Drosophila* glass vials for one hour prior to the bioassay and were individually tested at the olfactometer. The type of choice and time required to choose were recorded. A positive choice was scored when a fly entered at least two-thirds of an olfactometer arm and remained there for at least five seconds. The olfactometer was rotated by 180° after every five observations and thoroughly cleaned with soap and water, rinsed with analytical grade acetone, and air dried under a fume hood. For each exposure route-insecticide combination, at least 30 replicates were carried out, each consisting of one insect that made a choice.

2.5. Electroantennography (EAG)

The electroantennogram recordings were performed to measure the antennal sensitivity of mated *D. suzukii* females sublethally exposed to each insecticide LC_{10} by residual contact or larval ingestion. Each fly was individually immobilized within a 1000 µl disposable plastic pipette tip, with half of the head protruding from the truncated narrow end. Recording and reference electrodes were prepared by inserting a silver wire into a glass capillary tubes pulled with a micropipette puller (PC10 puller®, Narishige, Japan) and filled with 0.1 M KCl electrically conductive solution (Schiestl and Marion-Poll, 2002). MP22® micromanipulators (Syntech, Germany) were used to insert the reference electrode into the mouthparts beneath the proboscis, while the recording electrode was positioned in contact with the antennal tip. A stimulus controller (CS 55®, Syntech, Germany) delivered a humidified, charcoal-filtered airstream and three 1.0 s pulses of *Droskidrink* over the insect head. For stimulus preparation, 100 µl of *Droskidrink* were pipetted to a 6 × 0.5 cm strip of filter paper placed inside a 14.5 cm glass Pasteur pipette (Thermo Fisher Scientific, USA), with each pipette replaced after testing five insects. For each insecticide and exposure route, the maximum depolarization amplitude (-mV) was recorded from 29 to 40 mated females. Voltage variations were amplified and recorded using IDAC−4® (Intelligent Data Acquisition Controller, Syntech, Germany), and signals were analyzed with EAG2000® software (Syntech, Germany).

2.6. Transcriptomic analysis

Quantitative Real-Time PCR (qRT-PCR) was used to assess the expression of nine chemosensory-related genes coding for odorant receptors (*Or*) in mated *D. suzukii* females following sublethal LC_{10} exposure by adult residual contact or larval ingestion. The selected genes (*Or13a*, *Or42b*, *Or43b*, *Or46aB*, *Or67a4*, *Or67b*, *Or7a*, *Or85b*, and *Or98a*) were chosen based on their previously reported differential expression after mating (Crava et al., 2019).

For each insecticide and exposure route, total RNA was extracted from five mated females using the SV Total RNA™ Isolation System (Z3100, Promega Corporation, Madison, WI, USA), following the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop™ Lite Spectrophotometer (ND-LITE-PR, Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA synthesis was performed using 500 ng of total RNA and a Reverse Transcription System kit (A1250, Promega Corporation, Madison, WI, USA). The transcript levels were quantified by qRT-PCR using *Rpl32* and *Hsp83* as reference genes. Primer sequences are listed in Supplementary Table S2. Amplification reactions were conducted in a final volume of 10 µl containing 5 µl of SensiFAST™ SYBR® & Fluorescein mix (2x), 0.4 µl of each gene-specific primer (10 mM), and 1 µl of cDNA template. Thermal cycling conditions were 95°C for 120 s, followed by 40 cycles of 95°C for 5 s, 60°C for 10 s, and 72°C for 5 s. Reactions were carried out on AriaMx Real-Time PCR System (Agilent Technologies, USA). Three independent biological replicates, each with three technical replicates, were conducted for each treatment.

2.7. Data analysis

Raw datasets from the olfactometer, electrophysiological, and transcriptomic bioassays were tested for normality and homoscedasticity using the Kolmogorov-Smirnov and the Cochran tests, respectively. Preference data obtained through the Y-tube olfactometer bioassays were analyzed using a generalized linear model (GLM) with a binomial distribution and logit link function to assess the effects of the factor *insecticide* (four insecticides and untreated control), *exposure route* (residual and ingestion), and their interaction (*insecticide* × *exposure route*) on the probability of choosing the bait versus air. In addition, for each route of exposure and insecticide, a chi-squared goodness-of fit-test was used to determine whether choices (air vs bait) differed significantly from a 50:50 distribution.

A two-way analysis of variance (ANOVA) was used to assess the effects of *insecticide* (four insecticides and untreated control), *exposure route* (residual and ingestion), and their interaction (*insecticide* × *exposure route*) on (i) time for choice, (ii) EAG recordings, and (iii) *Or* gene expression. In addition, one-way ANOVA was used to analyze the insecticide effect for each exposure route. When significant differences were detected ($p \leq 0.05$), least significant differences (LSD) post-hoc tests were applied. Gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ normalization method (Livak and Schmittgen, 2001). All analyses were performed at a 95% confidence level using the software IBM SPSS Statistics for Windows version 26.0 (IBM Corp. 2011. Armonk, NY: IBM Corp.).

3. Results

3.1. Insecticide sublethal effects on orientation behavior

The GLM analysis showed that both exposure route ($\chi^2_1 = 4.54$; $p = 0.033$) and insecticide ($\chi^2_4 = 28.7$; $p < 0.001$) had significant effects on the probability of choosing the bait versus air, whereas their interaction was not significant ($\chi^2_4 = 2.84$; $p = 0.585$). A significant attraction towards the bait (*Droskidrink*) was expected for insects that did not experience any insecticide sublethal exposure, and this expectation was confirmed in the control treatments for both residual contact ($\chi^2_1 = 19.2$;

$p < 0.001$) and ingestion ($\chi^2_1 = 22.5$; $p < 0.001$) exposure routes.

After contact exposure, the preference of *D. suzukii* mated females for the bait over clean air was maintained in *D. suzukii* mated females exposed to cyazypyr ($\chi^2_1 = 16.1$; $p < 0.001$), or lambda-cyhalothrin ($\chi^2_1 = 4.80$; $p < 0.03$). In contrast, flies exposed to chlorpyrifos ($\chi^2_1 = 2.13$; $p = 0.14$) or spinosad ($\chi^2_1 = 4.33$; $p = 0.07$) showed no significant preference, indicating disorientation (Fig. 1A). Conversely, all the insecticides altered attraction of *D. suzukii* mated females toward the bait following larval ingestion of contaminated diet, resulting in no significant differences between choices for bait and clean air (chlorpyrifos: $\chi^2_1 = 0.13$; $p = 0.71$; cyazypyr: $\chi^2_1 = 0.13$; $p = 0.71$; lambda-cyhalothrin: $\chi^2_1 = 1.20$; $p = 0.27$; spinosad: $\chi^2_1 = 1.20$; $p = 0.27$) (Fig. 1B).

The time required by *D. suzukii* mated females to choose between bait (*Droskidrink*) and clean air was significantly affected by the factor *insecticide* ($F_4 = 2.89$; $p = 0.02$), but not by the *exposure route* ($F_1 = 0.07$; $p = 0.79$) (Table 1). However, a significant *insecticide* × *exposure route* interaction ($F_4 = 2.69$; $p = 0.03$) indicated that insecticide effects were dependent on the exposure pathway. Residual contact exposure of *D. suzukii* adults to lambda-cyhalothrin and chlorpyrifos significantly increased time needed to make a choice by 38.9% and 25.9%, respectively ($F_{4,145} = 3.53$; $p = 0.01$) (Fig. 2A). In contrast, following larvae sublethal exposure through ingestion, no insecticide significantly affected the time needed to make a choice of adult *D. suzukii* females ($F_{4,145} = 1.98$; $p = 0.10$) (Fig. 2B).

3.2. Insecticide sublethal effects on Drosophila suzukii EAG response

The two-way ANOVA showed that the effect of the factor *insecticide* (four insecticides and control) did not significantly affect EAG response (i.e., maximum depolarization amplitudes, -mV) ($F_4 = 0.84$; $p = 0.50$). In contrast, the *route of exposure* (residual vs ingestion) significantly influenced the antennal response ($F_1 = 4.46$; $p = 0.03$), independently of the insecticide factor, as indicated by the non-significant insecticide-exposure route interaction ($F_4 = 1.71$; $p = 0.15$) (Table 1). Results from the one-way ANOVA indicated that residual exposure significantly reduced the physiological olfactory perception of *D. suzukii* ($F_{4,169} = 2.78$; $p = 0.03$). Lambda-cyhalothrin caused the largest reduction (31%), followed by chlorpyrifos (27%) and spinosad (24%) (Fig. 3A). No

Table 1
Statistics from the two-way ANOVA used to analyze (A) the time required by *Drosophila suzukii* mated females to choose the bait (*Droskidrink*) in the Y-tube olfactometer (time for choice), (B) the maximum depolarization amplitudes (-mV) recorded from *D. suzukii* mated females during electroantennography (EAG response), and (C) the expression of the nine genes encoding odorant receptors in *D. suzukii* mated female antennae (*Or* gene expression), as affected by tested insecticides (insecticide factor) route of exposure (route of exposure factor), and their interaction (insecticide × route of exposure). Asterisks indicate significant differences at p≤ 0.05.

Source of variation	Degrees of freedom	F	*p*-value
(A) Time for choice			
insecticide	4	2.89	***0.023**
route of exposure	1	0.07	0.789
insecticide × route of exposure	4	2.69	***0.031**
(B) EAG response			
insecticide	4	0.84	0.501
route of exposure	1	4.55	***0.034**
insecticide × route of exposure	4	1.71	0.148
(C) *Or* gene expression			
insecticide	4	4.25	***0.003**
route of exposure	1	74.9	***< 0.001**
insecticide × route of exposure	4	4.75	***< 0.001**

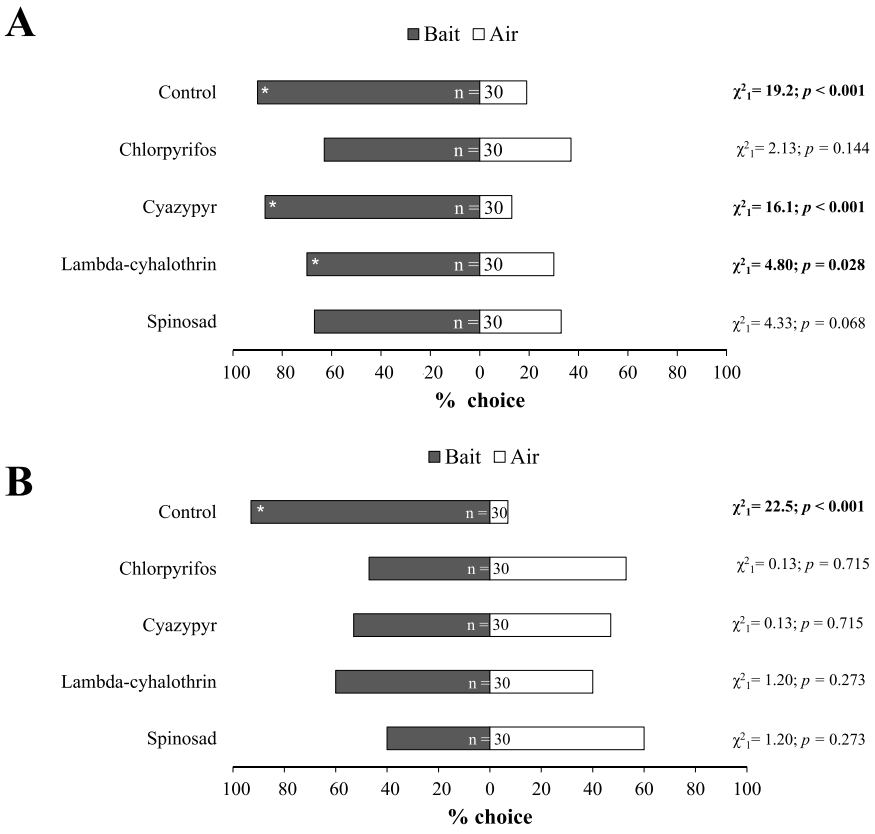

Fig. 1. Olfactory response (% of choices) of *Drosophila suzukii* mated females towards the bait (*Droskidrink*) and clean air after exposure to the insecticide LC_{10} by (A) residual contact as adults or (B) ingestion of contaminated artificial diet during larval development. n = number of *D. suzukii* mated females making a choice within the 5 min of observations. Statistics in bold indicate significant differences in the attraction toward the bait (*Droskidrink*) and clean air (likelihood chi-squared test $p \leq 0.05$).

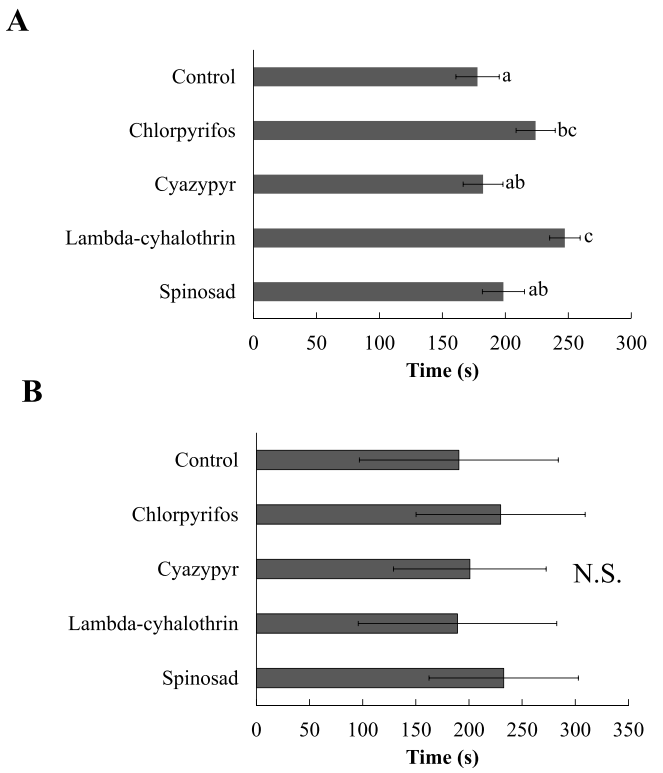

Fig. 2. Mean (±SE) time required (s) by *Drosophila suzukii* mated females to choose between the bait (*Droskidrink*) or clean air at the Y-tube olfactometer after exposure to insecticide LC_{10} by (A) residual contact as adults or (B) ingestion of contaminated artificial diet during larval development. Whitin each subfigure, different letters indicate significant differences among insecticides according to one-way ANOVA followed by LSD post hoc test for multiple comparisons ($p \leq 0.05$). N.S.: differences not statistically significant after one-way ANOVA ($p > 0.05$).

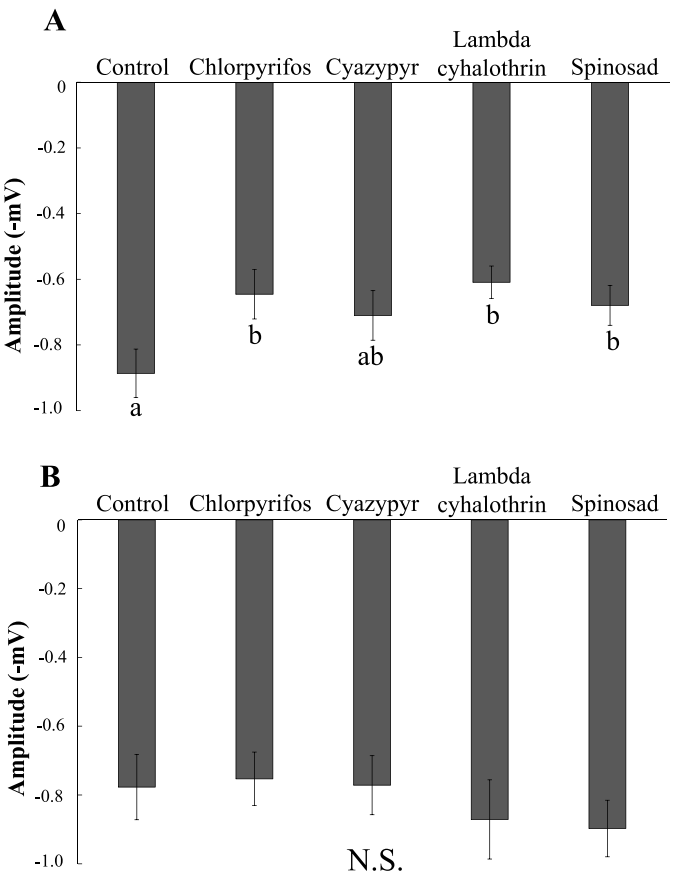

Fig. 3. Means (±SE) values of the maximum depolarization amplitudes (-mV) recorded by EAG on *Drosophila suzukii* mated females exposed to insecticide LC_{10} by (A) residual contact as adults or (B) ingestion of contaminated artificial diet during the larval development. Whitin each subfigure, different letters indicate significant differences among insecticides according to one-way ANOVA followed by LSD post hoc test for multiple comparisons ($p \leq 0.05$). N.S.: differences not statistically significant after one-way ANOVA ($p > 0.05$).

significant changes were detected in the olfactory responses of *D. suzukii* following larval exposure through insecticide ingestion ($F_{4,156}$= 0.53; $p = 0.71$) (Fig. 3B).

3.3. Insecticide sublethal effects on OR gene expression

The factor *insecticide* (F_4= 4.25; $p = 0.003$), *exposure route* (F_1= 74.98; $p < 0.001$), and their interaction (F_4=4.75; $p = 0.001$), significantly affected *Or* gene expression (Table 1). Following residual contact exposure of adults, all insecticides downregulated the expression of the nine *Or* genes analyzed in *D. suzukii* mated females. The lowest expression levels were consistently observed for *Or46ab*, Or67a4, *Or67b, Or7a,* and *Or85b*. Overall, lambda-cyhalothrin and spinosad exerted the strongest effects on *Or46ab* and *Or67a4*. Chlorpyriphos, followed by cyazypyr, showed comparatively weaker effects on *Or* gene expression (Fig. 4A). In contrast, the expression of adult *Or* genes was not significantly affected when *D. suzukii* was exposed to insecticides through larval ingestion (Fig. 4B).

4. Discussion

Neurotoxic insecticides can impair insect sensory systems by acting as information disruptors (Tricoire-Leignel et al., 2012), thereby altering olfactory-mediated orientation in *D. suzukii.* This study investigated the multifaceted sublethal effects of four neurotoxic insecticides on the orientation of *D. suzukii* mated females exposed either by residual contact as adults or by ingestion during larval development. Our results demonstrate that exposure route is an important determinant of olfactory-related behavioral outcomes. Larval insecticide ingestion caused a stronger disruption of adult orientation behavior than residual exposure. Conversely, adult residual exposure induced clear physiological and transcriptomic alterations in the antennae, which were not detected following larval ingestion. Under residual exposure, all insecticides at LC_{10} significantly downregulated *Or* gene expression, indicating a reduced capacity to synthesize functional olfactory receptors. With the exception of cyazypyr, a concomitant decrease in antenna sensitivity was detected through EAG. These findings are consistent with previous research showing that pesticide-induced changes in EAG amplitudes can result from alterations in the number and regulation of activated *Or* genes (Ke et al., 2023; Roat et al., 2022; Tricoire-Leignel et al., 2012). For instance, Tatarko et al. (2023) found that imidacloprid significantly reduced *D. melanogaster* odor response through inactivation of an olfactory sensory neuron (OSN) expressing the odor receptor *Or22a*. This OSN also expresses nicotinic acetylcholine receptor (nAChR) subunits Dβ1 and Dβ2, which are known targets of neonicotinoids (Tatarko et al., 2023). Together, these observations support the hypothesis that neurotoxic insecticides can directly impair *Drosophila* olfactory system, even at doses well below recommended field concentrations.

The high sensitivity of acetylcholinesterase (AChE) and nicotinic acetylcholine (nACh) receptors to chlorpyrifos (AChE inhibitor) and spinosad (nAChR modulator), even at low doses, may explain these effects (Salgado and Saar, 2004; Tatarko et al., 2023). Consistent with these physiological and transcriptomic responses, chlorpyrifos and spinosad also impaired behavioral orientation of *D. suzukii* females under

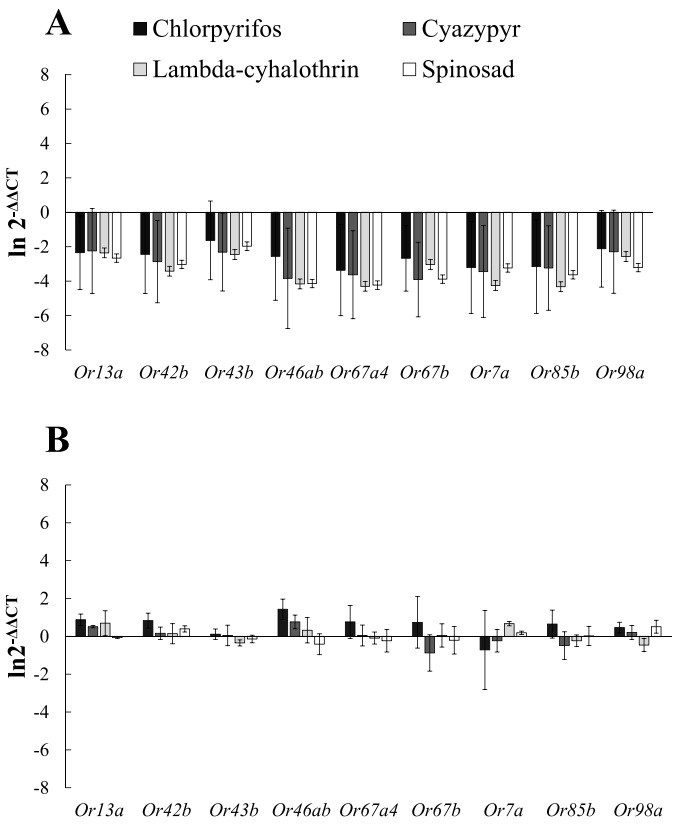

Fig. 4. Expression levels (mean ± SE) of nine genes encoding olfactory receptor (*Or*) in *Drosophila suzukii* mated females exposed to the insecticide LC_{10} by (A) residual contact as adults or (B) ingestion of contaminated artificial diet during the larval development.

residual exposure. Similar effects were observed in *Nesidiocoris tenuis* (Reuter) (Heteroptera: Miridae), where chlorpyrifos disrupted attraction and increased decision-making time (Passos et al., 2022). The lack of behavioral impairment under lambda-cyhalothrin, despite reduced *Or* expression and EAG responses, suggests partial preservation of receptor functionality or downstream compensatory mechanisms sufficient to maintain orientation.

Interestingly, the increase in decision-making time observed in *D. suzukii* following exposure to lambda-cyhalothrin and chlorpyrifos at LC_{10} points to a broader disruption in neuronal information processing, possibly involving central nervous system (Chen et al., 2024; Lisi et al., 2025; ZB Zhang et al., 2017). Comparable effects have been described in sex pheromone processing, where neurotoxic insecticides altered antennal lobe activity without directly affecting peripheral olfactory sensitivity (Rabhi et al., 2016). In addition, several studies have documented pesticide-induced behavioral alterations in *D. suzukii*, including changes in resting, grooming, walking, feeding and flight activities (Guedes et al., 2018, 2020). In contrast, cyazypyr did not alter antennal sensitivity or behavioral orientation, which is consistent with its primary mode of action. Diamide insecticides act as ryanodine receptor modulators, inducing uncontrolled calcium release in muscle tissues and leading to muscular paralysis without directly impairing synaptic neurotransmission (Casida and Durkin, 2013). In this context, the lack of detectable effects on antennal sensitivity or behavioral orientation may be even more evident at sublethal concentrations, such as the LC_{10} tested in the present study. Indeed, these concentrations are likely insufficient to substantially affect peripheral olfactory signaling pathways, unlike insecticides directly targeting cholinergic neurotransmission, such as chlorpyrifos and spinosad.

When *D. suzukii* adult females were exposed to insecticides via larval ingestion, significant adult disorientation in the olfactometer was observed, despite the absence of detectable effects on *Or* gene expression and EAG amplitudes. Because orientation toward an odor source involves the integration of multiple sensory and motor inputs (Visser, 1988), this behavioral disruption was likely not driven by periphery olfactory damage. This interpretation is reinforced by the fact that EAG measures only overall antennal sensitivity (Crava et al., 2019; Roelofs, 1984), whereas olfactometer-based orientation relies on complex neural integration and decision-making processes (Visser, 1988). We therefore hypothesize that larval exposure induces long-lasting neurophysiological alterations, potentially affecting neural processing, motor coordination, or cognitive function, ultimately disrupting olfactory-guided behavior in adults (Guedes et al., 2026).

Our previous study demonstrated pronounced alterations in key life history traits of *D. suzukii* following larval exposure to the same insecticides, including prolonged development time and reduced pupal size (Lisi et al., 2023). These effects were likely induced by hormonal dysregulation and impaired of neuronal tissue formation (Guedes et al., 2026). Similarly, Smith et al. (2020) showed that insecticide exposure during brood or early adult development reduced brain growth and impaired adult learning in bumblebees. In *D. melanogaster,* sublethal larval exposure negatively affected adult locomotion and orientation, altering activity levels, sleep patterns, circadian rhythms, and mating behavior (Young et al., 2020). Several additional studies showed that sublethal larval exposure to neurotoxicants induces neuronal apoptosis and impairs cognitive functions, including olfactory learning and memory, in adult honeybees (Chen et al., 2023; Tan et al., 2015, 2017; Vázquez et al., 2024; Yang et al., 2012). However, research on early-life insecticide exposure remains largely limited to behavioral endpoints, with few studies integrating physiological and transcriptomic approaches. Reported outcomes range from significant impairment (Ke et al., 2023) to no detectable effects (Wang et al., 2011). Despite its limited use, this integrative framework provides critical insight into delayed and carry-over effects of pesticide exposure, which are highly relevant in an eco-evolutionary context and for pesticide risk assessment (Young et al., 2020).

In conclusion, our findings emphasize the central role of exposure routes in shaping sublethal insecticide hazards. Residual contact exposure to chlorpyrifos and spinosad disoriented *D. suzukii* adults, whereas compensatory mechanisms may preserve olfactory function under lambda-cyhalothrin and cyazypyr exposure. In contrast, larval ingestion of all insecticides caused adult behavioral disorientation, likely reflecting broader and persistent neurophysiological disruption. Such multipathway exposure to low but environmentally relevant LC_{10} may induce neurobehavioral disruption not detectable by conventional mortality-based risk assessments and may represent a greater ecological hazard than currently predicted in real agroecosystems.

In this context, chlorpyrifos, spinosad and lambda-cyhalothrin may compromise IPM strategies based on olfactory cues, reducing trapping efficiency and interfering with attract-and-kill and push-and-pull approaches (Tait et al., 2021). Disrupted orientation may further affect host and mate localization, with potential ṕopulation-level consequences. Further studies should explore insecticides with diverse modes of action and identify specific OSNs and neural circuits affected, thereby supporting the development of IPM strategies that minimize interference with chemical communication.

CRediT authorship contribution statement

Carmelo Cavallaro: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Mariangela Milordo:** Investigation, Formal analysis, Data curation. **Daniele Cornara:** Writing – review & editing. **Antonio Gugliuzzo:** Writing – review & editing, Methodology. **Michele Ricupero:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Formal analysis, Data curation. **Gianfranco Anfora:** Writing – review & editing. **Marica Scala:** Investigation, Formal analysis, Data curation. **Antonio Biondi:**

Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Fabrizio Lisi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Guedes Raul N. C.:** Writing – review & editing. **Gaetano Siscaro:** Writing – review & editing, Supervision. **Nicolas Desneux:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2026.120380.

Data availability

Data will be made available on request.

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