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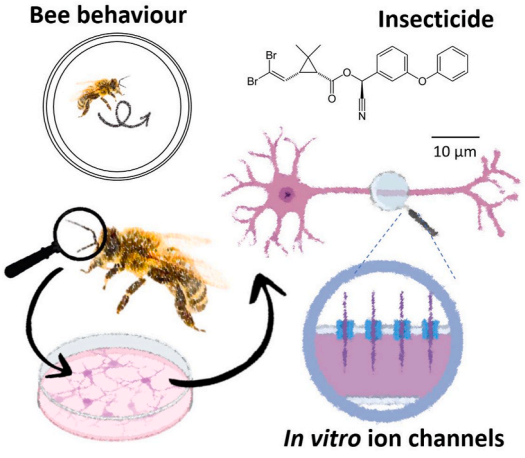
HIGHLIGHTS

- *In vitro* characterization of NaV-targeting insecticides to anticipate behavioral intoxication symptoms.
- Towards an *in vitro* prediction to replace mortality tests on living honey bees.
- A strategy in line with the principles of the 3Rs.

GRAPHICAL ABSTRACT



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ABSTRACT

The current risk assessment framework for insecticides suffers from certain shortcomings in adequately addressing the effects of low doses on off-target species. To remedy this gap, a combination of behavioural assays and *in vitro* cellular approaches are required to refine the precision of toxicity assessment. The domestic honey bee has long been standing as an emblematic pollinator in ecotoxicology, and once more, it provides us with a practical testing model for this purpose. First, newly emerged bees (D1) were found more vulnerable than 6 days-

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old bees (D6) to deltamethrin, a widely used α-cyano-3-phenoxybenzyle pyrethroid. In D1 bees, the range of doses inducing mortality was shifted towards lower values (~2-fold) with a correspondingly lower LD_{50} (11 ng/bee). Moreover, at low doses that do not induce mortality in laboratory conditions, the locomotor behaviour of D1 bees was more impacted than in D6 bees. This was evidenced by an increase in immobility time and a decrease in locomotor performance across all tested doses for D1 bees (0.75, 1.5 and 3 ng/bee) during automated 21 h-long observations. Behavioural disorders are linked to deltamethrin's disruption of voltage-gated sodium channels (Na_Vs) functions, as quantified in cultured neuronal cells. In the presence of deltamethrin, patch-clamp experiments revealed a concentration- and a use-dependent slowing of Na_V kinetics. Channel's deactivation is slowed by three orders of magnitude at 10 μM deltamethrin. Two additional phenoxybenzyle pyrethroids, including the commonly used cypermethrin, elicited quantitatively similar effects on Na_V kinetics. The integration of *in vitro* cellular assays and behavioural assays may facilitate a deeper understanding and prediction of insecticides toxicity.

1. Introduction

Recent decades have seen a worldwide decline in insects and pollinators, with harm to biodiversity and environment (Goulson, 2019; Hallmann et al., 2017; Jactel et al., 2020; Ollerton et al., 2014; Powney et al., 2019; Wagner et al., 2021). Insecticides are important contributors to this decline (LeBuhn and Vargas Luna, 2021; Sanchez-Bayo and Wyckhuys, 2019). In 2018, insecticides represented $18.4 billion USD in sales, with two major classes, pyrethroids and neonicotinoids, accounting for 16% and 25% in market share, respectively (Sparks and Bryant, 2021). In the European Union (27 member states), 2019 neonicotinoids active substance tonnage decreased significantly after usage of this class of insecticides has been restricted, according to data provided by Eurostat (2024). Conversely, volumes of pyrethroids increased 2-fold between 2011 and 2021 to reach volumes close to 1800 tons. In France for instance, volumes increased by 45% over the same period of time to reach 419 tons of pyrethroids sold (Food and Agriculture Organization, 2024). In this country, pyrethroids accounted for 34% of insecticides tonnage sold in 2021 (419 out of 1221 tons) if one excepts the non-neurotoxic insecticides such as kaolin and oils (6279 out of 7501 tons, (BNV-D Traçabilité, 2023)). Deltamethrin, tau-fluvalinate, lambda-cyhalothrin, and cypermethrin are the most used α-cyano-3-phenoxybenzyle pyrethroids for plant protection in France. Over the past decade (2011–2021), sales were close to 12, 35, 43 and 162 tons on average for these four compounds, respectively, with a steep increase for cypermethrin (50–200 tons, BNV-D Traçabilité, 2023)). These molecules have been proven highly toxic to honey bees with LD_{50}s ranging between 2 and 108 ng/bee for deltamethrin, 20–120 ng/bee for cypermethrin and 83–98 ng/bee for lambda-cyhalothrin (Poquet et al., 2014).

Regulations have been implemented worldwide in an effort to mitigate and ultimately reduce the use of insecticides (for instance, European Commission, 2009; Fourrier et al., 2022). For bees, most risk assessment tests focus on the lethal effects of insecticides (OECD, 2017a, 2017b, 2013, 1998a, 1998b for acute toxicity, and OECD, 2017c for chronic toxicity). This emphasis creates a knowledge gap regarding the sublethal effects of insecticides. There have been multiple attempts to close this gap with reports of insecticides impeding bees learning performance (Decourtye et al., 2005), homing flight (Decourtye, 2022; Henry et al., 2012) and locomotion (Collet et al., 2022). Novel tests, for instance the OECD Guidance N° 332 on homing flight (OECD, 2021) were suggested to be included in the risk assessment framework. Teeters et al. (2012), Ingram et al. (2015), and Charreton et al. (2015) demonstrated that a locomotion test can effectively detect behavioural effects of insecticides at low doses. Additionally, this test offered the advantage of being readily useable across multiple bee's developmental stages.

Modern toxicological approaches (the "3R" rule) put a particular emphasis on reducing and replacing animal usage with *in vitro* and *in silico* methods, with the ultimate aim of predicting the effect of insecticides on exposed organisms. *In vitro* approaches can include methods such as electrophysiology, and can be leveraged to better understand the functionality of the nervous system (as it is the main target of current insecticides), and the interaction of the insecticides with their molecular targets. Identified molecular targets for pyrethroids include the voltage-activated sodium, calcium and chloride channels, the ligand-gated receptors such the GABA receptors, and the nicotinic acetylcholine receptors (for review, see Clark and Symington, 2012; Soderlund, 2012). So far in bees, the most studied targets for pyrethroids are the neuronal voltage-activated sodium channels (Na_Vs) responsible for the action potentials, and exposure to pyrethroids is associated with an alteration in the transition of Na_Vs from their open state to and from their close state (Gosselin-Badaroudine et al., 2015; Kadala et al., 2011, 2014, 2019). *In vitro* approaches and *in silico* modelling have been proved effective in characterizing the molecular effect of pyrethroids in bee neurons (Kadala et al., 2014). By combining *in vitro* tests with locomotion assays, we can cover a larger spectrum of the effects of insecticides on the honey bee, ranging from cellular impacts to behavioural changes. This approach can help bridge the knowledge gap indicated previously. Therefore, in the current study, we used video-tracking on living bees and cell electrophysiology to investigate the correlation between behavioural changes induced by the pyrethroid deltamethrin in *Apis mellifera* and its effects on neuronal and muscular functions.

2. Materials and methods

2.1. Acute toxicity test

Honey bees (*Apis mellifera*) were collected from colonies maintained by beekeepers in the research department apiary. The contact toxicity of deltamethrin was assessed after a single exposure. Technical-grade deltamethrin (CAS Number 52918-63-5) was dissolved in acetone at concentration 10 μg/μl to form the stock-solution. Deltamethrin purity was >98%. Doses depicted in Results are not corrected for the level of purity. Doses of interest (0, 0.75, 1.5, 3, 6, 12, 25, 50, and 100 ng/μl) were obtained with serial dilutions in acetone, starting with the stock-solution. All solutions were stored at −20 °C in amber glass vials until usage. Three groups of ten bees placed in standard plastic cages were used for each dose. Newly emerged (D1) and 6-days old (D6) bees were anaesthetized with CO_2 for 30 and 60 s, respectively prior to exposure to deltamethrin. Using a Hamilton syringe, 1 μl of solution with the desired dose was deposited on the dorsal side of the thorax. Exposed bees were then placed in a ventilated incubator (29 °C, 40% relative humidity, dark) and were provided with water and Candi sugar paste (85% sucrose, 5% glucose, 3% fructose, water; Candi Apifonda, Icko-Apiculture) *ad libitum*. Bees' mortality was monitored for 24 h following exposure to deltamethrin.

The acute toxicity test allowed us to determine the sublethal dose 24 h after exposure (SLD_{24h}). This is the dose that produces a mortality level not statistically different from the control, while a dose 2 times higher than this SLD_{24h} causes mortality significantly higher than the control. For the locomotor capacity test, we used a range of doses that were compliant with these criteria. These doses were validated using Fisher's exact tests in GraphPad Prism version 6.

2.2. Locomotor performance

For the locomotor test, after exposure to low doses described above and before bees awakening from anaesthesia, they were individually transferred into individual locomotor arenas, made of clean plastic Petri dishes (diameter 50 mm, depth 15 mm; Thermo Fisher, Rochester, NY). Individual bee's body weight was measured. A feeder filled with Candi sugar paste *ad libitum* was placed on the right lateral region of each arena. Arenas were inserted in a perforated clear plastic tray placed vertically 4 cm in front of a red screen (a computer monitor with colour settings set to red only). A webcam (Microsoft LifeCam, 640x480 pixels) placed 40 cm away from the screen, allowed for the monitoring of bees' displacements. The monitor, the plastic tray and the webcam were enclosed in an opaque climatic chamber (Model #3744, Forma Scientific Inc., Marietta, Ohio) to maintain constant temperature (29 °C) and avoid influence of daylight variations. The webcam was operated with Image J software (Rasband, 1997), through a custom-made plugin. Image calibration was systematically set before the start of the experiment and the resolution of 1.65 pixels/mm was sufficient to capture bees walking activity accurately (bee size ~ 14 × 5 mm). Before starting detection of bees' movements, the plugin allowed for the extraction of a background image that will be subtracted online afterwards. The custom plugin performed background subtraction on each grabbed image, and this was followed by an image binarization (after a threshold step). Particle detection was used to track the movement of individual bees in circular regions of interest defined around each arena. The plugin detected the coordinates of each bee (particle centroid) at a frequency of 20 Hz. Instantaneous distance was calculated, and the distance d covered from the start of monitoring was computed. Every 10 min, the value of d (as well as its timestamp value) was automatically written in a text file for further offline treatment. At the end of the 21 h observation period, 126 successive values of d (and timestamps) were thus used to calculate offline, the average speed (S) of each bee at every 10-min interval. A threshold of 3 mm/s was set as the limit for an actual walking activity and only average speeds above this threshold were considered. Values of S were then used to recalculate the actual total distance D covered by each bee. This experimental setup allows for the measurement of 24 bees at a time and per computer. Three computers were used simultaneously for each experiment on D1 and D6 bees.

2.3. Cell isolation

Neurons were extracted from prepupae antennae (9–11 days of preimaginal development) and dissection was performed following a protocol described previously (Kadala et al., 2011). Neurons were maintained *in vitro* in Petri dishes coated with poly-L-lysine and filled with culture medium (see Solutions). Antennal neurons were used for experiments within three days *in vitro*.

Muscle fibers were isolated from newly emerged bees following a procedure previously established (Collet, 2009). Briefly, bees were anaesthetized with cold (4 °C) before heads were cut out and metathoracic legs collected. Tibias from these legs were cut longitudinally and kept open with fine minuten pins in a calcium-free Tyrode (see Solutions). Enzymatic dissociation was performed at 37 °C for 20 min in a cocktail consisting of collagenase type I (0.5 mg/ml), trypsin (1.5 mg/ml), papain (1.5 mg/ml), and protease (1 mg/ml), all dissolved in a calcium-free Tyrode. The preparation was then rinsed with calcium-free Tyrode and then transferred in a Tyrode solution supplemented with $CaCl_2$ (2 mM). The final step was the gentle trituration of muscle masses with a pipette and their dispersion in a Petri dish containing extracellular solution (see Solutions section).

2.4. Solutions

The culture medium for neurons isolation consisted in a liquid Leibowitz L-15 solution containing L-glutamine (Sigma-Aldrich, St. Quentin-Fallavier, France) and supplemented with FBS (foetal bovine serum, 10%), penicillin/streptomycin (1%), L-proline (3.3 mM), and D-glucose (5.5 mM). Tyrode solution for dissection contained (in mM): 140 NaCl, 5 KCl, 10 HEPES (N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)). For muscle fibers, the Tyrode solution for dissection was supplemented with 2 mM $MgCl_2$.Osmolarities for these solutions were adjusted with sucrose to 400 mOsm/l for neurons and to 300 mOsm/l for muscle fibers, and pH was 7.2.

Solutions for patch-clamp were designed for the recording of currents through sodium channels or calcium channels in neurons and in muscle fibers, respectively. Consequently, standard extracellular solution for patch-clamp in neurons contained blockers of calcium and potassium channels and contained (in mM), 120 NaCl, 20 TEA-Cl (Tetraethylammonium chloride), 2 $MgCl_2$, 2 $BaCl_2$, 0.1 $CdCl_2$, 1 4-aminopyridine, 10 HEPES, (pH 7.2, 400 mOsm/l). Intracellular solution contained (in mM): 135 CsCl, 5 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES, 90 sucrose, pH 7.2 (380 mOsm/l). For recordings in muscle fibers, the extracellular solution contained (in mM): 140 TEA-$MeSO_3$ ($MeSO_3$, Methanesulfonic acid), 2 $MgCl_2$, 2 $BaCl_2$, 1 4-AP, 10 HEPES, pH·7.2 (300 mOsm/l). Intracellular solution contained (in mM): 140 K-gluconate, 2 $MgCl_2$, 10 EGTA, 10 HEPES, pH·7.2 (290 mOsm/l). All solutions for cell isolation and electrophysiology were sterile-filtered through a 0.2 μm filter.

A stock solution of deltamethrin at 10 mM was prepared in DMSO (dimethyl sulfoxide) and then dissolved in the perfusion solution at the target concentration. DMSO concentration never exceeded 0.1%. Cypermethrin, permethrin and tetramethrin were prepared as deltamethrin and had a purity of >95%, >98% and >98%, respectively. Compounds were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France). Muscle fibers were exposed to deltamethrin through a gravity-driven perfusion system (perfusion rate was 0.1 ml min^{-1}) described earlier (Collet and Belzunces, 2007). Antennal neurons were perfused using a single-use glass capillary system as described by Tatebayashi and Narahashi (1994). Only one neuron or muscle fiber was recorded in any given Petri dish that was perfused with deltamethrin. The perfusion system and reference electrode were thoroughly rinsed with running 70% ethanol and distilled water after each series of experiments. All experiments were performed at room temperature (20–25 °C).

2.5. Patch-clamp recordings

A patch-clamp amplifier (RK400, Bio-Logic, Claix, France) was used in the whole-cell configuration to measure membrane currents. Recordings were made under voltage-clamp mode. Voltage pulse generation and data acquisition were done using WinWCP software (John Dempster, Strathclyde University, UK) driving an A/D-D/A converter (PCI-6014 board, National instruments Corp. Austin, TX, USA). Patch-clamp pipettes were pulled from borosilicate glass capillaries on a vertical pipette puller (P30, Sutter Instrument Co, Novato, CA, USA). Sylgard-coated (761036-1 EA, Sigma-Aldrich, St. Quentin-Fallavier, France) electrodes were used to minimize the residual pipette capacitance that was compensated for analogically. The resistance of microelectrodes filled with intracellular solution (see Solutions) ranged between 6 and 10 MΩ for neurons, and were 3 MΩ on average in standard extracellular solutions for muscle fibers. Microelectrode offset potential was nulled prior to seal formation and residual microelectrode capacitance was zeroed out with an analogue compensation circuit available on the amplifier after gigaseal (seal resistance >1 GΩ) formation but before membrane patch rupture. The resting holding potential was set to −80 mV prior to the transition to the whole-cell configuration. Capacitance and series resistance were compensated for in neurons. Only series resistance was compensated for in muscle fibers. For neurons stimulation, we used either a single or repeated depolarizations (test pulses) at 35 Hz consisting of 3-ms stimulations (from a resting potential of −80 mV to −10 or 0 mV) with an inter-pulse (duration between the initiation of two successive test pulses) of 28

ms. Passive leak currents and residual linear capacitive currents were subtracted using a P/4 protocol. The effects of pyrethroids on voltage-gated sodium channels are traditionally quantified by estimating the percentage of modified channels with the following equation: $\%M = [(I_{tail} \div (E_h - E_{Na})) \div (I_{Na} \div (E_t - E_{Na}))] \times 100$, where M is the percentage of modified channels, I_{tail} is the maximal tail current amplitude upon repolarization, E_h is the potential to which the membrane is repolarized, I_{Na} is the amplitude of the sodium current (measured in control conditions) during depolarization, E_t is the membrane potential reached during the test pulse and E_{Na} is the equilibrium potential of the sodium ion (Tatebayashi and Narahashi, 1994). I_{tail} was measured 3 ms after the end of the depolarizing pulse. Muscle fibers currents were obtained with a 400-ms voltage ramp from −80 to +40 mV. Passive membrane currents were subtracted using a P/4 protocol.

Statistical analyses were performed with GraphPad Prism (GraphPad Prism version 6 for Windows, GraphPad Software). For neurons, the distribution was tested with the Shapiro-Wilk test and data were analysed accordingly either with Student's, Mann-Whitney or Wilcoxon tests. For multiple comparisons, we used the Kruskal-Wallis test associated with Dunn's post-test. For data from muscle fibers, we used the Student's test. We considered statistical significance for $p < 0.05$. Data are presented as mean ± s.e.m throughout the text.

3. Results

3.1. Toxicity and behavioural effects induced by deltamethrin at low doses

Deltamethrin was very toxic to bees, both at D1 and D6. In the (collective cage) mortality assay, most D1 and D6 bees died quickly after exposure to 100 ng deltamethrin per bee (94% and 100%, respectively at 24h). No mortality was recorded at all in control conditions (acetone only) for both D1 and D6 bees as they tolerated caging remarkably well. The dose causing 50% mortality (LD_{50}) was determined as the inflection point of a logistic sigmoidal function fitted to experimental data. LD_{50}s were 11 ng/bee and 19 ng/bee for D1 and D6 bees, respectively, 24 h after exposure to deltamethrin (Supplementary Fig. S1). A global ~2-fold shift in the dose-mortality curve towards lower doses suggested a higher susceptibility of D1 bees, a trend that was confirmed when we compared mortality at dose 12 ng, close to the sigmoid's inflection point, for the two ages (67% for D1 versus 23% for D6, $p = 0.0038$, Fisher's exact test).

Rather than just surveying the well-known lethal effects of deltamethrin on bees, one objective of the present study was to assess consequences of this molecule at low doses. These are doses that do not cause mortality but rather behavioural changes that could compromise individual life traits, with substantial consequences at the colony level. To study the effects of low doses of deltamethrin on bees' behaviour, non-lethal levels were chosen at the leftward foot of the dose-mortality sigmoidal relationship. Doses above 1.5 ng/bee for D1 bees and 6 ng/bee for D6 bees caused mortality levels significantly higher than the respective control conditions ($p < 0.01$, Fisher's exact test). For D1 bees, 3 ng/bee caused a significant 23% mortality ($p = 0.0003$, Fisher's exact test) while no mortality was observed for 1.5 ng/bee and below. For D6 bees, doses 3 and 6 ng/bee were sublethal after 24 h (maximum 7% mortality, not statistically different from control, $p > 0.5$, Fisher's exact test) and dose 12 ng/bee induced a significant mortality (23%, $p = 0.0053$, Fisher's exact test). In practice, behavioural effects of deltamethrin were monitored in individual locomotor arenas for 21 h for the following low doses: 0.75, 1.5 and 3 ng for D1 bees and 1.5, 3 and 6 ng for D6 bees.

In individual locomotor arenas, experimental doses remained sublethal for 21 h ($p > 0.3$, Fisher's exact test). Even the dose 3 ng was found sublethal for D1 bees in these conditions ($p = 0.2898$, Fisher's exact test). Through the 21 h of continuous automatic observation, D1 bees were half as active when exposed to deltamethrin. Indeed, control D1 bees spent on average 45 ± 6 % of their time in activity, whereas bees exposed to 0.75, 1.5 and 3 ng deltamethrin spent 25 ± 3, 26 ± 4 and 24 ± 5% of their time active, respectively (Fig. 1A, $p < 0.05$, Mann-Whitney test). Interestingly, control bees had more variability, with an activity rate ranging from 15 to 83% of their time from one bee to another. At 3 ng, one D1 bee (out of 18) had an exceptional behaviour, with a hyperactive profile as compared with the other deltamethrin modalities. On average, bees exposed to 0.75 and 1.5 ng deltamethrin did not compensate for their lower activity rate by walking at higher speed (Fig. 1B, $p < 0.05$, Mann-Whitney test). Bees exposed to 0.75 and 1.5 ng indeed covered only half of the distance covered by control bees. Again, at 3 ng deltamethrin, 3 bees (out of 18) showed a hyperactive profile as compared with bees exposed to the two other deltamethrin modalities and even as compared with control.

Qualitatively similar results were obtained with older bees (D6). It is known that division of labour in hives is dependent on bees' age, due to anatomical/physiological/behavioural differences. At age D6, bees start to forage in natural conditions. Remarkably, D6 bees had an average body weight 26% lower than D1 bees (82 ± 1 mg and 111 ± 1, $n = 69$, respectively; $p < 0.0001$; Mann-Whitney test). Despite this anatomical difference, in the absence of deltamethrin, D6 bees had a motricity equal to D1 bees, with a total distance covered in 21 h of 449 ± 50 m (Fig. 1D) and 583 ± 94 m (Fig. 1B), respectively ($n = 15$, $p = 0.6160$, Mann-Whitney test). D1 and D6 bees were also equally active, with 45 ± 6 and 36 ± 3% of their time spent active, respectively ($p = 0.5599$, Mann-Whitney test, Fig. 1A and C). While no major behavioural differences were found between D1 and D6 bees in control conditions, D6 bees were

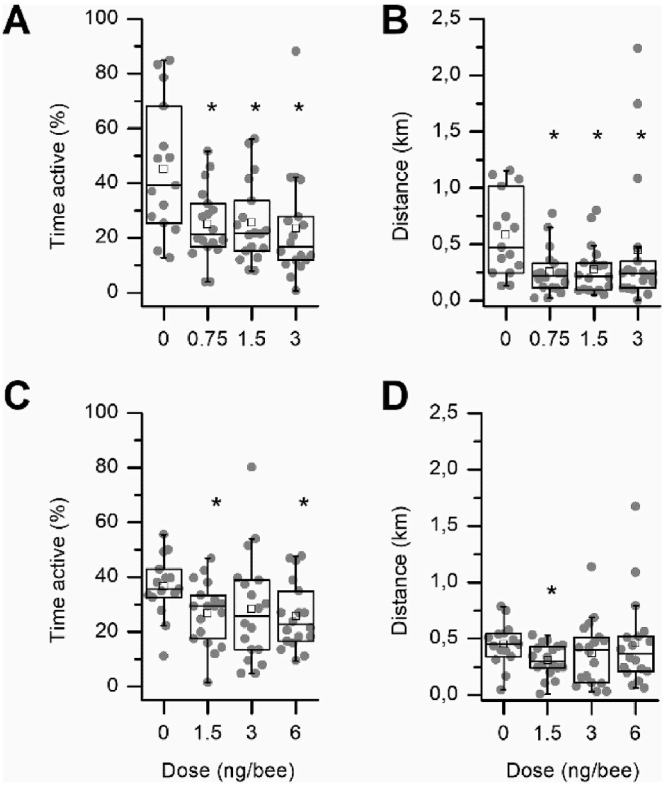

Fig. 1. Bees exposed to low doses of deltamethrin show a decrease in locomotor performance. (A) D1 bees exposed to 0.75 through 3 ng/bee of deltamethrin are half as active as their control counterparts and travel significantly less distance (B) with instances of hyperactivity at dose 3 ng/bee. (C) D6 bees exposed to deltamethrin 1.5 and 6 ng/bee are less active as compared with control bees and the lowest dose causes bees to underperform in terms of distance travelled (D). Some D6 exposed bees show a hyperactive profile at 3 and 6 ng/bee. For both D1 and D6 bees, sample sizes are 15 for control and 18 for each deltamethrin dose.

however found more susceptible to claustration in the individual locomotion arena than D1 bees. A low mortality was observed in control conditions during the 21 h locomotion period (2 out of 15 bees for D6 but 0 out of 15 bees for D1). Control mortality level for D6 bees was not significantly higher than in-cages assay described above at the same age ($p = 0.9262$, Fisher's exact test). This is also true for all three doses of deltamethrin. Mortality rates were similar as well across all exposure conditions (including control) in locomotion arenas ($p > 0.4$) confirming that these doses remained sublethal during the locomotion assay. As compared with control D6 bees, bees exposed to doses 1.5 and 6 ng had a significant decrease in the percentage of activity for 21 h (by a factor 1.4) to 27 ± 3 and 26 ± 3% of time, respectively (Fig. 1C, $p < 0.05$). The same tendency was seen at 3 ng deltamethrin, but this effect was not statistically significant. In D6 bees, the total distance covered decreased significantly by a factor 1.5 with 1.5 ng deltamethrin (Fig. 1D, $p = 0.022$). However, this trend was not significant for higher doses. As it was also the case at D1, hyperactivity was clearly observable at D6 at the two highest doses tested (6 ng, 2 bees out of 18, Fig. 1D). Deltamethrin sublethal doses thus had the general effect of decreasing activity in exposed bees although instances of hyperactivity were observed in a subset of bees especially at the highest doses tested (*i.e.* 3 ng for D1 bees and 6 ng for D6 bees).

Behavioural alterations in bees may come from molecular action of deltamethrin on the neuromuscular system. In particular, neuronal voltage-gated sodium channels (Na_Vs) are known as the primary targets of pyrethroid insecticides (Kadala et al., 2019).

3.2. Deltamethrin slows down neuronal voltage-gated sodium channels leading to an abnormal long-lasting sodium current

Here for the first time, we thoroughly describe major changes induced by deltamethrin on bee's Na_Vs activation and deactivation kinetics. In our patch-clamp recordings, a fast-activating and fast-inactivating sodium current develops under control conditions upon a single depolarization of 3 ms duration (Fig. 2A, arrowhead). The membrane potential was stepped to −10 mV from a holding potential of −80 mV and the effects of deltamethrin were assessed 3 min after the beginning of exposure. The most noticeable effect of deltamethrin under these conditions is the tail current that appears upon repolarization (Fig. 2B, arrowhead).

This reflects the abnormal delay in channel's deactivation in the presence of deltamethrin. Development of the tail current is associated with a decrease in sodium peak current amplitude which is significant at 10 μM (−39%, $n = 8$, $p = 0.0392$, unpaired *t*-test) but not at 1 μM (−26%, $n = 9$, $p = 0.1186$, unpaired *t*-test), as compared with control ($n = 17$, Fig. 2C).

3.3. Effect of deltamethrin on antennal neurons' sodium channels is both activity-dependent and concentration-dependent

Deltamethrin is known to better bind on Na_Vs in their open configuration (Vais et al., 2000). This phenomenon is traditionally described as the use-dependence of pyrethroids' action. We used a series of short depolarizing pulses (3 ms, 35 Hz) to mimic trains of action potentials during a neuronal activity, and recorded the resulting sodium current under control condition (Fig. 3A, top trace). Perfusion with deltamethrin at 1 or 10 μM caused a cumulative tail current to develop upon repolarizations (Fig. 3A, middle and bottom traces). This observation denotes the fact that deltamethrin can bind to sodium channels in open state.

We also looked at the changes in the relative sodium peak amplitude in response to repetitive depolarizations. Successive amplitudes were normalized to the amplitude of the sodium current during the first depolarization. In control condition, the current peak amplitude tended to decrease as a result of a population of sodium channels transitioning to an inactivated state. On average, this decrease was 11% ($n = 13$, $p = 0.0134$, Wilcoxon matched-pairs signed rank test) between the first and the tenth depolarization (Fig. 3B, hollow circles). Deltamethrin 1 μM stabilized the sodium peak amplitude (Fig. 3B, grey circles) with only 1% change between the first and the tenth depolarization ($n = 9$, $p = 0.8737$, Student's paired *t*-test). Concentration 1 μM was not sufficient to induce use-dependence. On the contrary, with deltamethrin 10 μM, there was a progressive increase in the peak current amplitude along with repeated depolarizations. On average, this increase was 37% ($n = 8$, $p = 0.0888$; Student's paired *t*-test) between the first and the tenth depolarization (Fig. 3B, black circles). As compared with control, multiple depolarizations with deltamethrin 10 μM caused a use-dependent increase in sodium peak amplitude beginning from the fifth depolarizing pulse ($n = 13$ for control and $n = 8$ for deltamethrin 10 μM; $p < 0.01$, unpaired *t*-test).

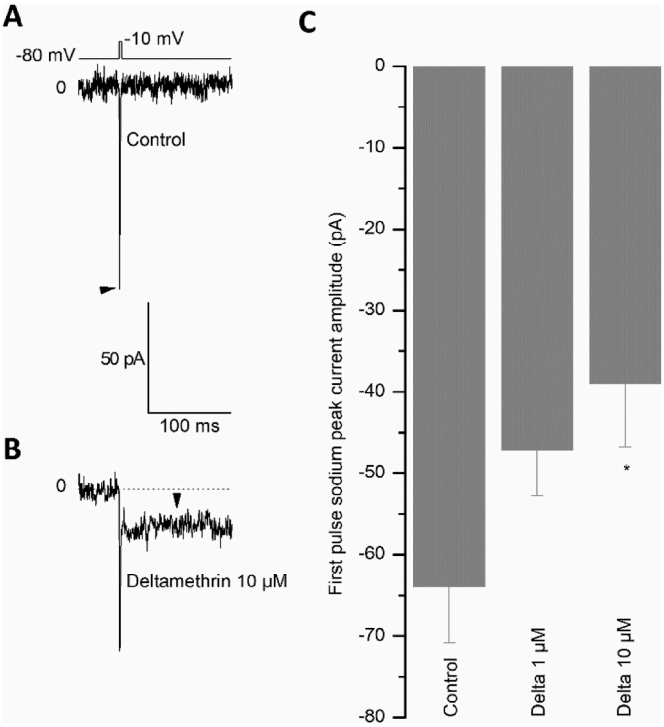

Fig. 2. Deltamethrin alters the deactivation kinetics of bee's Na_Vs. (A) An example of sodium current recorded in control conditions in response to a single 3 ms depolarization from −80 to 10 mV (top trace). Arrowhead shows the peak sodium current. (B) Deltamethrin 10 μM induces a tail current upon repolarization (arrowhead), reflecting a slowdown in the deactivation phase of the sodium channel. (C) Sodium peak current amplitude significantly decreases with deltamethrin at 10 μM but not at 1 μM, as compared with control. *$p < 0.05$.

The amplitude of the tail current reflects the proportion of sodium channels which are bound to deltamethrin. This proportion is expressed as a percentage, as detailed in section 2.5. For both concentrations, the proportion of modified channels increased with successive depolarizations but this proportion was more important with deltamethrin at 10 μM than at 1 μM, regardless of the number of depolarizations considered ($p < 0.05$, unpaired *t*-test, Fig. 3C). Upon the first depolarization, deltamethrin 1 μM modified 3 ± 1% of channels ($n = 9$) and this percentage rose significantly after ten repeated depolarizations to 15 ± 2% of channels ($n = 9$, $p = 0.0001$, Student's paired *t*-test). At concentration 10 μM, deltamethrin modified 9 ± 3% of channels ($n = 8$) at the first depolarizing pulse and a much higher proportion (38 ± 11%, $n = 8$, $p = 0.0193$, Student's paired *t*-test) at the tenth.

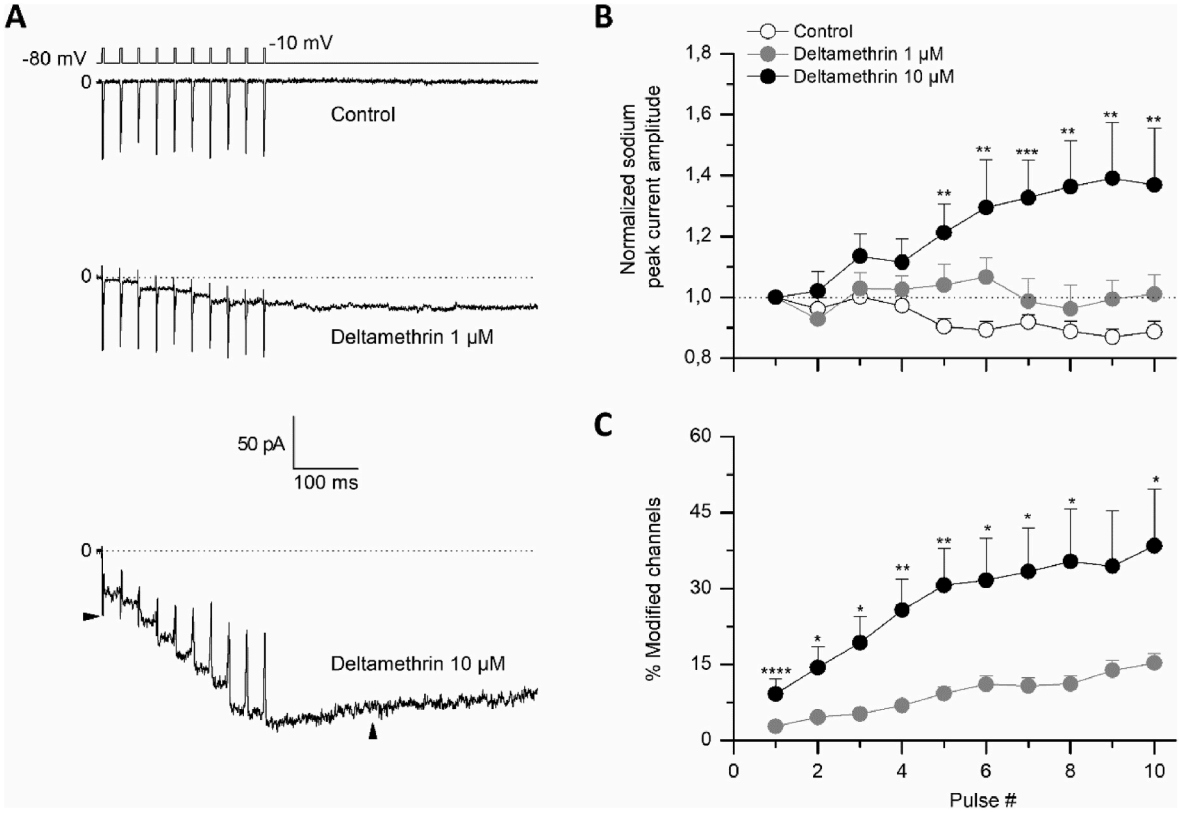

Fig. 3. Effects of deltamethrin on sodium currents are both use-dependent and concentration-dependent. (A) Examples of voltage-gated sodium current recorded in response to a series of ten depolarizations from −80 to −10 mV at a frequency of 35 Hz (shown above). Unlike the control conditions (top trace), deltamethrin 1 μM (middle trace) and 10 μM (bottom trace) induce a cumulating tail current. Arrowheads show the sodium peak current (left) and the sodium tail current (right). (B) Evolution of the peak current along with repetitive depolarizations as shown in (A). Amplitudes are normalized to the amplitude at the first depolarization. Peak current amplitude slightly decreases along the successive depolarizations in control conditions (white circles) but stabilizes with deltamethrin at 1 μM (grey circles), and significantly increases with deltamethrin 10 μM (black circles). (C) The percentage of modified sodium channels increases as a result of the cumulating tail current. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.4. Persistence of deltamethrin-induced tail current

Another proxy to characterize the pyrethroid effect is to measure the tail current persistence. This persistence reflects the delay in channel's closure in the presence of pyrethroid. To approximate the decay kinetics, we measured the residual tail current right after the tenth pulse as well as 600 ms later. The ratio (R_{600}) obtained from these two values provides us with an estimation of the proportion of channels still open after 600 ms. With deltamethrin 10 μM, R_{600} was 45 ± 11% (n = 6, Fig. 4A). This means that nearly half of the exposed sodium channels are still open 600 ms after the end of the depolarization, whereas in control conditions all channels close in less than 1 ms.

In another set of experiments, the R_{600} was measured for three other pyrethroids in conditions similar to deltamethrin (10 μM for tetramethrin, permethrin, or cypermethrin, structure illustrated in Fig. 4B). The R_{600} was 71 ± 7% (n = 6) for cypermethrin, 26 ± 7% (n = 8) for permethrin, and 3 ± 2% (n = 8) for tetramethrin.

Deltamethrin, cypermethrin, and permethrin were not significantly different from one another with regard to R_{600} (p > 0.05, Kruskal-Wallis test associated with Dunn's post test), indicating a similar slowdown in channel's shutting for the three molecules. Kruskal-Wallis multiple comparisons also show that R_{600} for tetramethrin was statistically different from deltamethrin (p = 0.0141) and cypermethrin (p = 0.0007) but not from permethrin (p = 0.122).

Locomotor deficits observed on bees, although originating from strong neural effects on Na_Vs could also come from additional direct effect on muscular cells excitability.

3.5. Deltamethrin has no significant effect on voltage-gated calcium channels from the skeletal muscle fibers

Some pyrethroids have been shown to change current density through voltage-gated calcium channels (Ca_Vs) and/or their voltage dependence, in the absence of abnormal tail current (Collet, 2009; Hildebrand et al., 2004; Symington and Clark, 2005). Ca_Vs are responsible for action potentials in bee muscles (Collet and Belzunces, 2007). This prompted us to test the effects of deltamethrin on the honey bee Ca_Vs from skeletal muscle fibers. To avoid cell contraction, we replaced calcium with barium as charge carrier.

We recorded I–V curves in response to a 400 ms-long ramp protocol. Calcium channels activated at −54 ± 2 mV (n = 6), maximal peak amplitude was recorded at −20 ± 3 mV (n = 6), and current reversed at 20 ± 8 mV (n = 6). In the presence of deltamethrin, tail currents which are typical of Na_Vs modification were never observed through Ca_Vs (not illustrated). Five minutes after the onset of 10 μM deltamethrin perfusion, no significant impact on the current density was detected: −3.6 ± 0.5 pA/pF (n = 6) for control and −4.1 ± 0.5 pA/pF (n = 6) for deltamethrin (Fig. 5A and B; p = 0.0625, Wilcoxon matched-pairs signed rank test).

While deltamethrin induces strong modification of bees neuronal Na_Vs, no significant effect was detected on muscle fibers Ca_Vs, suggesting that action of deltamethrin on Na_Vs underlie the toxicological effects that we observed at individual level.

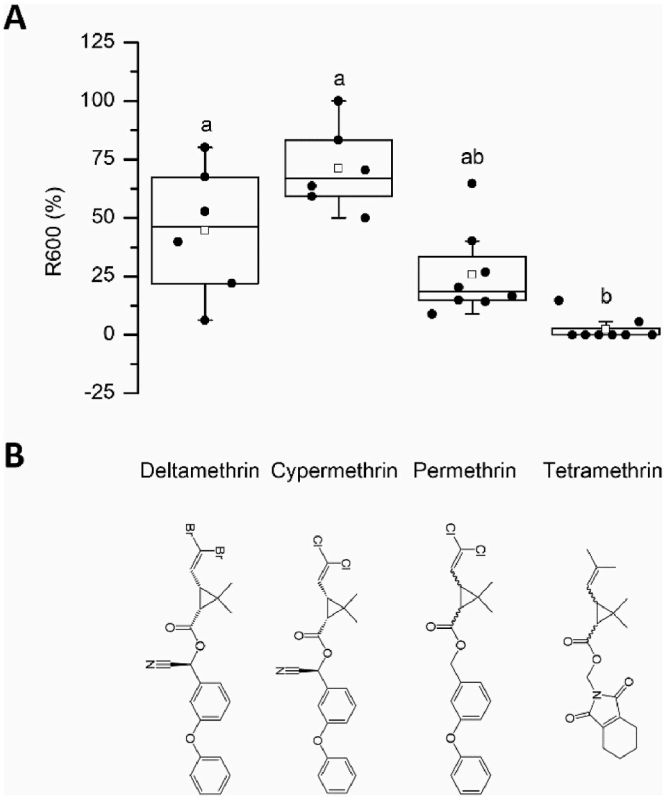

Fig. 4. Persistence of the pyrethroid-induced tail current. (A) The residual tail current 600 ms after the end of the tenth pulse (R_{600}) as a proxy for the decay of the tail current, is shown for each of the four pyrethroids. From left to right deltamethrin, cypermethrin, permethrin and tetramethrin. See text for further details. (B) Formulas for the four pyrethroids. Different letters represent statistically different values; $p < 0.05$.

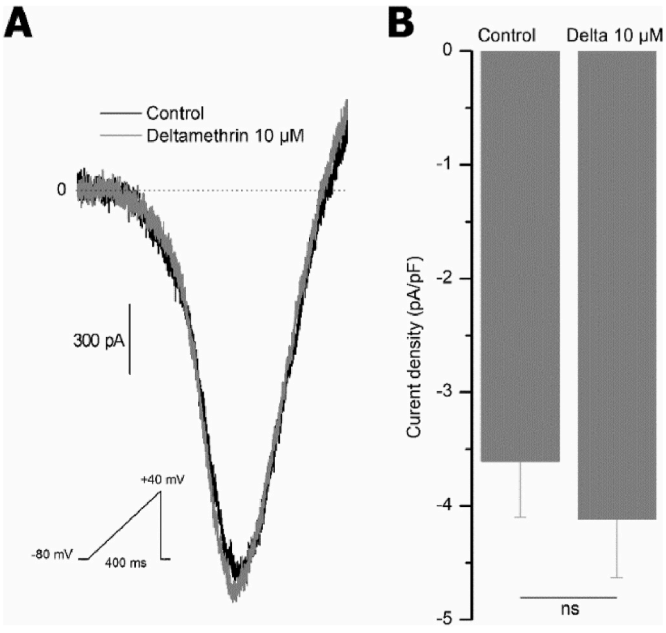

Fig. 5. Deltamethrin has no effect on muscle fibers voltage-gated calcium channels. (A) Current-voltage curve recorded in muscle fibers, in response to a 400 ms-long ramp protocol (bottom left, not at scale) in control (in black colour) and in the presence of 10 µM deltamethrin (in grey colour). The charge carrier in our experiments is barium. (B) Deltamethrin 10 µM has no effect on the current density. ns = not significant.

4. Discussion

In this work, the coupling of a behavioural test and an *in vitro* cellular assay sheds a new light on the toxicity of the insecticide deltamethrin for the adult honey bee. Our work characterizes for the first time, the deep kinetic changes operated by deltamethrin on bee Na_V channels. The data further document the link between molecular changes observed *in vitro* and behavioural effects at low doses (and mortality). These new data also further accredit the strategy of evaluating insecticides *in vitro* rather than on living bees, which concurs with modern toxicology strategies (the 3R rule: 'Refine, Reduce, Replace'). A major finding of this study is that deltamethrin is more toxic to younger bees as demonstrated both in classical mortality tests and behavioural locomotor tests. Whereas mandatory OECD tests required for market authorization focus on mortality only, another useful contribution of our work thus resides in the objective quantification of locomotor performances in individual arenas, leading to an accurate characterization of toxicological effects at low insecticide doses. The EFSA (European Food Safety Authority, 2023, 2013) reemphasized the need for behavioural tests in order to better evaluate insecticides toxicity towards beneficial insects. Candidate behavioural toxicological tests on bees were also reviewed recently (Collet et al., 2022; Fourrier et al., 2022). Here, in young bees, even after a single contact with a low dose that do not induce mortality, deltamethrin has debilitating effects on most bees as it hinders activity and locomotor performances. Effects are more complex in older bees as statistical effects are not observed in a consistent manner for all low doses tested. Interindividual differential effects may be responsible for this situation, with hyperactive behaviours revealed in a subset of bees. Further investigation will be needed to understand the basis of the interindividual differences but other insecticides have been shown to promote hyperactive or hypoactive locomotor activity depending on doses or time after exposure (Lambin et al., 2001). The higher sensitivity of young bees to deltamethrin recalls our previous results with another α-cyano-3-phenoxybenzyle pyrethroid: tau-fluvalinate. Newly emerged bees exposed to a sublethal dose of tau-fluvalinate experienced a ~2-fold stronger locomotor impairment than bees aged 4 days (Charreton et al., 2015; Teeters et al., 2012). Low doses of other α-cyano-3-phenoxybenzyle pyrethroids lambda-cyhalothrin, esfenvalerate and cypermethrin have been shown to induce −37 to −71% of locomotor performances (Charreton et al., 2015; Ingram et al., 2015). However, the locomotor impairment induced by low doses of pyrethroids is not specific to α-cyano-3-phenoxybenzyle pyrethroid since tetramethrin which structure is devoid of a phenoxybenzyle (replaced by a tetra-isoindole moiety) induces similar effects on bees (Charreton et al., 2015). Permethrin, which do not possess an α-cyano moiety, is quite potent as well (Ingram et al., 2015). Locomotor behaviour also proved to be a valid approach in evaluating sublethal effects of other major insecticide families (Collet et al., 2022) such as anthranilic diamides (Kadala et al., 2019), phenylpyrazoles (Aliouane et al., 2009; Charreton et al., 2015; El Hassani et al., 2005), neonicotinoids (Alkassab and Kirchner, 2018; Charreton et al., 2015; El Hassani et al., 2005; Lambin et al., 2001) or organophosphates (Williamson et al., 2013). Deltamethrin is widely used in the field at doses ranging from 5 to 20 g/ha and bees directly exposed to field treatment (spraying) may receive doses from 53 to 210 ng/bee (see calculation method in Poquet et al., 2014, PLoS ONE 9). Our dose-mortality data confirm that these doses are highly toxic to bees. Low doses tested in the present manuscript are environmentally relevant since deltamethrin doses of 23–39 ppb (2–4 ng/bee) have been described in the body of bees collected in hives (Mullin et al., 2010 PLOS ONE 5; Chauzat and Faucon, 2007 Pest Manag Sci 63). Our experiments show behavioural alterations after exposure to low deltamethrin doses. The higher-tier semi-field OECD test #75 contains an endpoint that may reflect sublethal behavioural effects, but this endpoint is hardly taken into account in the risk assessment process, due to its weakness. Indeed, the endpoint is obtained from a rudimentary assay consisting in counting the number of foragers on a 1 m^2 flower

patch during 15 s only. This assay cannot be seriously considered as objective, reproducible and representative of foraging performances. In the future, Method 75 needs to be refined, for instance by counting individual foragers trips numbers and trips durations with RFID chip or QR code counters (Decourtye et al., 2011 Ecotoxicology 20; Prado et al., 2019 Sci Total Environ 10). Counting the actual number of foragers on a flower patch with cameras during a sufficient amount of time would bring objective data as well (Ratnayake et al., 2021; PLoS One 16).

Locomotor deficits observed after exposure to low doses suggest an effect of deltamethrin on the neuromuscular system. We tested deltamethrin on both skeletal muscle fibers where action potentials are triggered by Ca_Vs (Collet and Belzunces, 2007), and in neurons where Na_Vs support the action potentials (Laurent et al., 2002; Schäfer et al., 1994). In muscle fibers, we did not detect any effect of deltamethrin on Ca_Vs. However, previous reports have shown a partial blocking effect of some pyrethroids on these channels both in vertebrates and bees. For instance, allethrin had a blocking effect on Ca_Vs in bee muscle fibers (Collet, 2009) and mammalian Ca_Vs expressed in an heterologous system (Hildebrand et al., 2004; Neal et al., 2010) without any effect on channel's deactivation (no tail current). Allethrin's action could be related to the presence of the oxo-cyclopentene moiety which is replaced in deltamethrin by a phenoxybenzyle moiety. Deltamethrin was shown to block rat's Ca_V expressed in *Xenopus* (Symington and Clark, 2005) while permethrin has a similar effect on flies' calcium channels (Yan et al., 2011). There is the untested possibility that in honey bees, deltamethrin interferes with neuronal calcium channels, additionally to Na_Vs.

To explore neuronal effects of deltamethrin, we have chosen peripheral neurons because they clearly represent a better cell model than central neurons with regard to their sensitivity to pyrethroids (Kadala et al., 2014). Our results show major effects of deltamethrin on the deactivation process of Na_Vs (tail current) and complement previous data showing modulation of voltage-dependence in central neurons (Zhou et al., 2011). Qualitatively, deltamethrin shows effects similar to other pyrethroids previously tested (cypermethrin, tetramethrin, permethrin; Kadala et al., 2019, 2014, 2011). Modern toxicology relies on *in vitro* methods in order to reduce quantities of organisms used. In the case of pyrethroids, the question is whether the molecular *in vitro* effects can be predictive of toxicity at individual level. In Kadala et al. (2014), the traditional index of molecular modification (percentage of modified channels) did not properly estimate the toxicity of pyrethroids. The percentage of modified channels induced with tetramethrin was much higher than with cypermethrin or permethrin, while tetramethrin is the least toxic for bees (worst case LD_{50}s from literature were 155, 20, 24 ng/bee for tetramethrin, cypermethrin and permethrin, respectively (Pesticide Properties DataBase, 2023; Poquet et al., 2014);).

Another approach to assess the functional modification of Na_Vs by pyrethroids is to consider the persistence of the tail current (R_{600}). The tail current for deltamethrin is at least three orders of magnitude slower than in control conditions and this molecule seems to be the most toxic of pyrethroids considered according to toxicity values from literature (worst case LD_{50} is 2 ng/bee (Pesticide Properties DataBase, 2023; Poquet et al., 2014),). Pyrethroids with similar LD_{50}s did not differ in R_{600} either, and high toxicity of the pyrethroids tested seem to correlate with a higher persistence of the sodium tail current. Therefore, R_{600} could be considered a good predictor of pyrethroids toxicity. From the structural point of view (see Fig. 4B), phenoxybenzyle-containing molecules (deltamethrin, cypermethrin, permethrin) show similar LD_{50} and R_{600} values whereas tetramethrin which harbours a tetra-isoindole moiety sets itself apart. Molecular effects can be at least partly ascribed to the physico-chemical properties of these compounds. For instance, tetramethrin is more soluble in water than permethrin, cypermethrin and deltamethrin (1.83, 0.011, 0.009, 0.002 mg/l for tetramethrin, permethrin, cypermethrin, and deltamethrin, respectively (Pesticide Properties DataBase, 2023)). Tetramethrin's solubility in organic solvents is much weaker than the 3 other compounds, suggesting that it is the least hydrophobic one. Furthermore, short term exposure is fully reversible for tetramethrin but not for permethrin, for instance (Kadala et al., 2011). This can contribute to tetramethrin being more easily removed from its Na_V target. Identification of amino acid substitution in Na_Vs of pyrethroid-resistant insects, site-directed mutagenesis, and computer models concurred in identifying two sites involved in pyrethroid binding (Dong et al., 2014; Du et al., 2013; Tan et al., 2002, 2005 Zhorov and Dong, 2016). Computer models suggest that in the case of deltamethrin and permethrin, the halogen atoms, the dimethylcyclopropane group and the phenoxybenzyle group help keep Na_Vs in an open state (Dong et al., 2014; Zhorov and Dong, 2022). Cypermethrin possesses these three moieties but although not specifically investigated, one can assume that its molecular interactions with Na_Vs are similar to deltamethrin and permethrin. Neither the halogen atoms nor the phenoxybenzyle group are present in tetramethrin. This would suggest that tetramethrin binds to Na_Vs in a less favourable fashion than deltamethrin, permethrin or cypermethrin, and possibly help explain the relatively rapid decay of its tail current and reversibility upon washout *in vitro*.

In conclusion, low doses of deltamethrin result in different effects on bees depending on age, with younger bees being more vulnerable than the older ones. At the molecular level, an *in vitro* measurement of the pyrethroid-induced tail current decay and its persistence could be a good indicator of pyrethroid toxicity levels for bees.

CRediT authorship contribution statement

Aklesso Kadala: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mahira Kaabeche:** Writing – original draft, Investigation. **Mercédès Charreton:** Writing – original draft, Investigation, Formal analysis, Data curation. **Jérôme Mutterer:** Software, Methodology. **Michel Pélissier:** Methodology. **Thierry Cens:** Writing – review & editing. **Matthieu Rousset:** Writing – review & editing. **Mohamed Chahine:** Writing – review & editing. **Pierre Charnet:** Writing – review & editing, Funding acquisition, Conceptualization. **Claude Collet:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143852.

Data availability

All data generated or analyzed during this study are included in this published article.

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