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Learning, memory, and sensory perception are impaired by exposure to the organophosphate, ethion, and the insect growth regulator, hexaflumuron, in honey bees (*Apis mellifera* L.)

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ABSTRACT

Insecticides are a major tool for controlling pest species. Their widespread use results in damage to non-targeted insects, with honey bees particularly at risk. During foraging, honey bees learn and remember floral characteristics that are associated with food. As insect pollinators, honey bees inadvertently contact chemicals which can have multiple negative impacts. The toxicity of two insecticides from different classes, ethion (47.79 mg a.i. L^{-1}) and hexaflumuron (500 mg a.i.L^{-1}), on learning, memory, and sensory perception were evaluated. We found that oral exposure to ethion had adverse effects on learned proboscis extension toward reward-associated odors and colors. In addition, we showed reduced sucrose consumption and sucrose responsiveness after exposure. Hexaflumuron also impaired olfactory learning and memory and decreased responsiveness to sucrose and water. Exposure to sub-lethal concentration of the cholinergic organophosphate insecticide, ethion (47.79 mg a.i. L^{-1}), and the field-recommended concentration of hexaflumuron (500 mg a.i.L^{-1}), significantly impaired behavior involved in foraging. Our results suggest that several behavioral characteristics of honey bees be evaluated when testing an insecticide rather than relying on just one behavioral measure.

Introduction

The decline of honey bee populations is a global concern (Mayack et al., 2022). Among the causes of population decline, insecticides are considered a major factor because of their lethal and sub-lethal effects. Sub-lethal effects are important to consider as they are difficult to detect and can cause colony dysfunction (Capela et al., 2022; Bartling et al., 2019; Crall et al., 2018). Cholinergic insecticides, for example, alter neuronal activity, lead to an imbalance between the production of reactive oxygen species affect the body's antioxidant defense systems, disrupt mitochondrial function, and ultimately lead to the death of neurons (Moffat et al., 2015; Peng and Yang, 2016; Martelli et al., 2020).

Due to the sub-lethal effects of insecticides on insect pollinators, the use of some insecticides is banned and restrictions have been imposed on their use (Stokstad, 2013; Butler, 2018; Goulson and Signatories, 2018). However, despite restrictions, there are still concerns about their widespread use (Sonne and Alstrup, 2019; Pyke, 2022).

Several studies have shown that pesticides from different groups such as neonicotinoids, organophosphates, and insect growth regulators can impair the learning and memory of honey bees (Al Naggar et al., 2015; Delkash-Roudsari et al., 2020a; Ludicke and Nieh, 2020a). Exposure to sub-lethal and field-realistic concentrations of insecticides can notably impair olfactory and visual learning (Abramson et al., 2012; Lundin et al., 2015; Tison et al., 2019; Delkash-Roudsari et al., 2020a; Delkash-Roudsari et al., 2020b; De Stefano et al., 2014). Therefore, pesticide-induced impairment of learning may disturb the fitness of colonies and possibly reduce their ability to pollinate (Lundin et al., 2015; Wu et al., 2022) as honey bees need to learn the shape, color, and odor of flowers to forage successfully (Dukas and Visscher, 1994; Menzel and Muller, 1996).

For honey bees, nectar serves as their main source of carbohydrates (Chalcoff et al., 2006). Sucrose, glucose, and fructose are the main components of nectar in flowers (Lindqvist et al., 2018), but bees prefer sucrose (Barker and Lehner, 1974). They learn very rapidly to associate nectar as a reward and flowers during foraging which is a key factor for foraging-related decisions (Barker and Lehner, 1974; Hill et al., 2001;

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Scheiner et al., 2004).

In the laboratory, honey bee learning is most often studied using a variation of the proboscis extension response technique (PER) in which restrained honey bees receive a pairing of a conditioned stimulus (CS) with an unconditioned stimulus (US, sometimes called a reward) (e.g. Bitterman et al., 1983). In such a paradigm a floral scent or color would serve as the CS and nectar (sucrose) would be the US (Jung et al., 2017).

In Middle Eastern countries such as Iran, few studies have investigated the effects of insecticides on sensory perception of water and sugar, or on visual and olfactory learning of honey bees. Here, we use the PER technique to focus on two commercial products available in the insecticide market in Iran – ethion, and hexaflumuron. In Iran, both of these insecticides are recommended as a means of combating pests of fruit and citrus trees during the spring season.

Ethion is used against aphids and scale insects in citrus orchards (Talebi-Jahromi, 2011). This insecticide is an organophosphorus insecticide that acts by inhibiting the cholinesterase enzyme in the nervous system of insects (Talebi-Jahromi, 2011). Hexaflumuron, an insect growth regulator or IGR, is a benzoyl urea compound that inhibits the synthesis of chitins. It has systemic effects, as well as being effective against Coleoptera, Lepidoptera, and Homoptera larvae (Talebi-Jahromi, 2011). Hexaflumuron is used on fruit trees, cotton, and potatoes (Talebi-Jahromi, 2011). In previous work on ethion and similar compounds (Abramson et al., 2004; Delkash-Roudsari et al., 2020), it had been found that ethion impairs aversive learning (Delkash-Roudsari et al., 2020), and that diflubenzuron (an IGR with the same mechanism of action as hexaflumuron) impairs PER learning (Abramson et al., 2004).

The primary objective of this research was to investigate whether ethion and hexaflumuron, have adverse effects on the learning and sensory perception of honey bees. We tested the sub-lethal effects of ethion and hexaflumuron on honey bee visual and olfactory learning and memory, and the sensory perception of water and sugar. Considering ethion's neurotoxic properties and hexaflumuron's role as a chitin synthesis inhibitor, we hypothesized that these insecticides could potentially induce a range of side effects, ultimately disrupting honey bee sensory perception and learning capacities.

Materials and methods

Honey bee samples

We used adult worker honey bees *Apis mellifera* L. (Hymenoptera: Apidae) of indeterminate age obtained from the College of Agriculture and Natural Resources, University of Tehran, Iran. The bees came from three hives maintained weekly and verified for queen health, forager activity, and eggs. Plastic cups were placed in front of the entrance of the hive, and when a significant number of workers entered the cup, the cup was sealed with mesh and transferred to the laboratory.

Insecticides and insecticide concentrations

Commercial products available in the insecticides market in Iran were purchased and used. The formulations contained: ethion (EC 47 %), and hexaflumuron (Consult®, EC 10 %). For ethion, the concentration tested corresponds to the LC_{30} (47.79 mg a.i.L^{-1}) according to the active ingredient, and for hexaflumuron, the field-recommended concentration (500 mg a.i.L^{-1}) according to the active ingredient was used. The reason why LC_{30} was used was that according to the source (Talebi-Jahromi, 2011), effects that cause less than 30 % mortality in the population of beneficial arthropods are known as sublethal effects. The final concentrations per bee were: Ethion LD_{30}: 477 ng bee $^{-1}$ and hexaflumuron LD_{30}: 5000 ng bee $^{-1}$(Delkash-Roudsari et al., 2022). Based on the method of Laurino et al., 2013, preliminary experiments were carried out to determine sub-lethal concentrations. The rationale behind using the recommended concentration of hexaflumuron is based on a prior study that demonstrated no obvious effects on the health of foragers. Therefore, in this research, we employed the field-realistic concentration of hexaflumuron to specifically investigate its potential side effects on honey bees.

Oral exposure of honey bees to insecticides

Experiments were performed on worker honey bees collected from the entrance of the hives. According to the EPPO Guideline 170 method (EPPO, 2001), the bees were placed in an incubator and deprived of food for 2 h. Following incubation, bees received 50 % sucrose solution containing insecticide concentrations or 50 % sucrose (w/v) solution without insecticide (control), in 2 ml microtubes for two hours.

For the PER conditioning and sensory perception tests, bees were anesthetized on ice and individually restrained in a plastic tube. To secure the bee to the tube, tape was used so that only its head, antenna, and mouthparts were exposed and moved freely. These restrained bees were placed for 3 to 4 h in a dark incubator at 35±1 °C and 50±5 % relative humidity to adapt to the new conditions. Each experiment (visual and olfactory learning, sucrose and water responsiveness, water and sucrose consumption), consisted of at least 40 adult worker honey bees with an untreated control group (fed sucrose only). The experiment was repeated three times.

Visual learning and memory test

The study was conducted according to Dobrin and Fahrbach (2012) with modifications. The conditioned stimulus was a blue LED light (light intensity 1090 lx). Following exposure to the insecticide, the tip of a toothpick was dipped into a 50 % sucrose solution and then used to stimulate the antenna. Only honey bees that showed a rigorous extension of their proboscis were included in subsequent conditioning experiments. Each bee was exposed to blue light for 8 s, and in the last 4 s, the toothpick was again used to stimulate the antennae. When the proboscis extended, the bee was allowed to lick the toothpick for 3 s. Ten conditioning trials were carried out and the interval between each trial was 10 min. One hour after the last conditioning trial a test trial was presented in which color alone was presented for 8 s. A condition response was recorded as a yes-or-no response. The test was done in a dark chamber (30×20×35) cm.

Olfactory learning and memory test

The procedure was similar to that used in the visual learning test. For olfactory PER conditioning, a toothpick soaked in a solution of sucrose at a concentration of 50 % was touched to the antennae of each restrained honey bee. Only honey bees that showed a vigorous extension of their proboscis were included in subsequent conditioning experiments. Conditioning trials were done with 10 Âµl of pure linalool as the CS. After oral exposure to the insecticide, each restrained bee was exposed to the linalool. The linalool was placed on a strip of filter paper and secured to the plunger of a 5 ml syringe (Abramson et al., 2012). Each bee was exposed to the CS odor for 6 s. After 3 s of odor delivery, the 50 % sucrose US was presented first to the antennae and now to the extended proboscis. It was allowed to lick the toothpick for 3 s.

Three conditioning trials were carried out and the interval between each trial was 10 min. Then, one hour after the last conditioning test, the conditioned PER was recorded as a yes-or-no response when the odor alone was delivered during the 6 s of a test trial (Decourtye et al., 2005; Al Naggar et al., 2015; Jung et al., 2017). We would like to note that we only used 3 trials because our concern is not with asymptotic performance but with the acquisition of the conditioned response.

Water and sucrose responsiveness

To test the effects of ethion (EC47%) and hexaflumuron (Consult®, EC 10 %) on water responsiveness, after oral exposure to the insecticides, the PER test was performed with water. To perform this test, the antenna of restrained honey bees was stimulated with water, and the PER was recorded as a yes-or-no response in the control and treatment groups.

To test the responsiveness of the PER to sucrose solution following insecticide treatment, the antennae of the bee were stimulated at 3-minute intervals of ascending concentrations of 0.03, 0.1, 0.3, 1, 3, 10, and 30 % (w/v), sucrose. The PER was recorded as a yes-or-no response in the control and treatment groups. By applying different concentrations of sucrose to the antennae of restrained bees, the sucrose responsiveness threshold for individuals was determined (Aliouane et al., 2009; Scheiner et al., 2004).

Water and sucrose consumption

After oral exposure, 50 % (w/v) sucrose solution without insecticide and water was provided to the honey bee for 24 h. The consumption of sucrose without insecticide and water was measured at 24 h (Aliouane et al., 2009).

Statistical analysis

Data analyses were performed with SPSS (version 22). The normality of data was assessed with a Kolmogorov–Smirnov test. The response to the conditioned stimulus was scored as a yes-or-no response and analyzed by Chi-Square (χ^2) tests. Statistical analysis for water and sucrose consumption was performed by comparing the means using a T-test. An alpha level of 0.05 was used.

Results

Visual learning and memory

Oral exposure to 50 % sucrose solution containing a sub-lethal concentration of ethion (47.79 mg a.i.L^{-1}) decreased visual learning (χ^2 = 48.85, P < 0.001) (Fig. 1A) and memory (χ^2 = 4.33, P = 0.03) (Fig. 1B) of treated bees compared to the untreated control, while hexaflumuron in the field-recommended concentration (500 mg a.i.L^{-1}) did not affect learning (χ^2 = 1.25, P = 0.26) (Fig. 1A) and memory (χ^2 = 2.21, P = 0.13) (Fig. 1B).

Olfactory learning and memory

Oral exposure to 50 % sucrose solution containing a sub-lethal concentration of ethion (47.79 mg a.i.L^{-1}) and hexaflumuron in the field-recommended concentration (500 mg a.i.L^{-1}) decreased olfactory learning (χ^2 = 4.37, P = 0.03; χ^2 = 7.48, P < 0.001 respectively) (Fig. 2A) and memory (χ^2 = 12.43, P < 0.001; χ^2 = 12.35, P < 0.001 respectively) (Fig. 2B) of treated bees compared to the untreated control.

Water and sucrose responsiveness

Oral exposure to 50 % sucrose solution containing a sub-lethal concentration of ethion (47.79 mg a.i.L^{-1}) did not affect water responsiveness (χ^2 = 0.51, P = 0.47) (Fig. 3A) of treated bees compared to the untreated control, while hexaflumuron in the field-recommended concentration (500 mg a.i.L^{-1}) decreased water responsiveness (χ^2 = 20.27, P < 0.001) (Fig. 3A) of treated bees compared to the untreated control.

A significant decrease in sucrose responsiveness by honey bees orally treated with ethion (47.79 mg a.i.L^{-1}) (χ^2 = 30.47, P < 0.001) (Fig. 3B) and hexaflumuron (500 mg a.i.L^{-1}) (χ^2 = 97.34, P < 0.001) (Fig. 3B) was

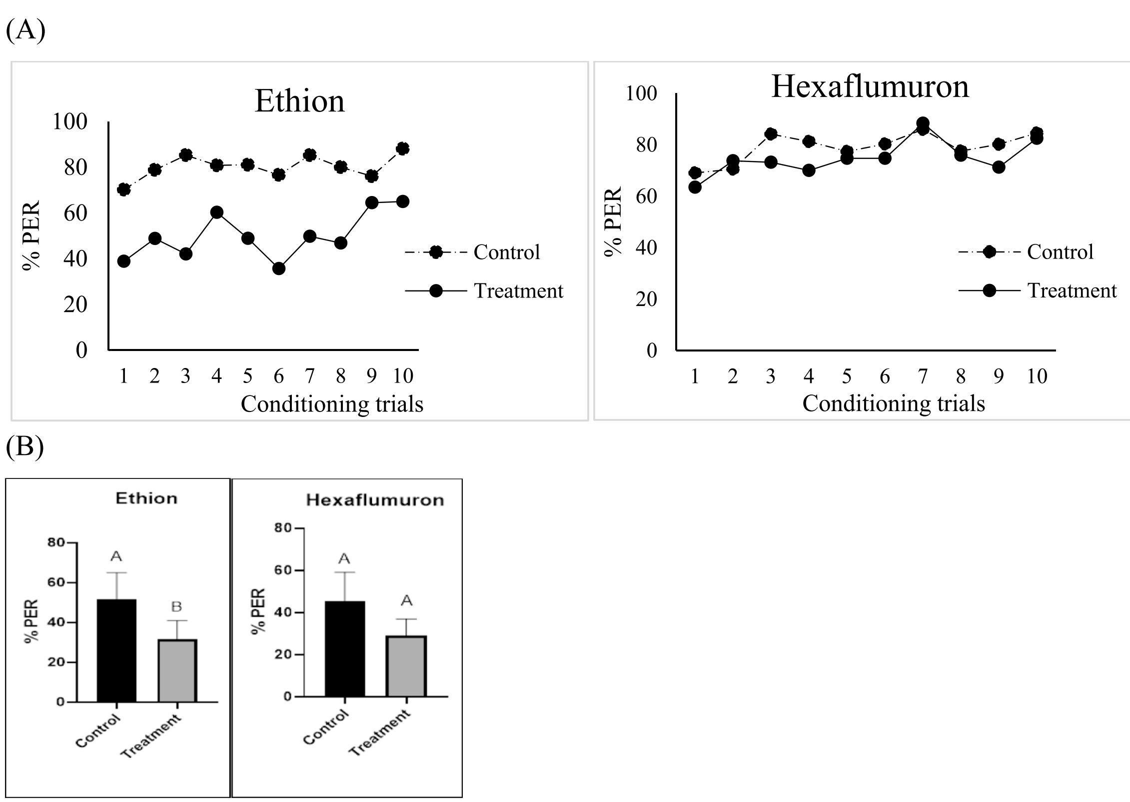

Fig. 1. Visual learning (A) and memory (B) performance measured as (%) (mean ± SEM) of worker honey bees exposed to an untreated control or ethion (79.47 mg a.i.L^{-1}), and hexaflumuron (500 mg a.i.L^{-1}) concentrations. The response of worker honey bees to the stimulus (CS and US) was analyzed by Chi-Square (χ2) tests. Different letters denote a significant difference between groups (A). The memory test occurred one hour after acquisition (B) (P < 0.05).

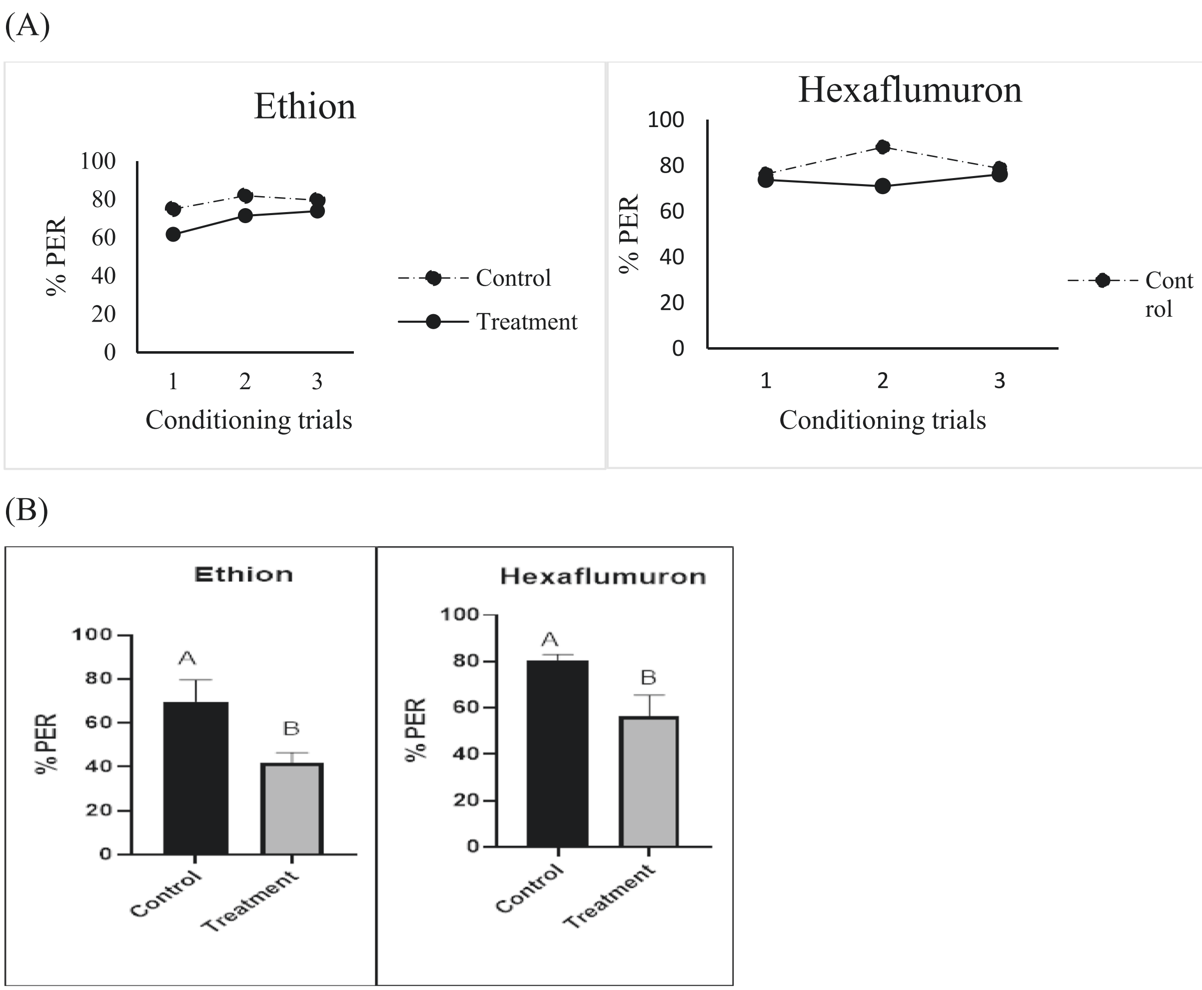

Fig. 2. Olfactory learning (A) and memory (B) performance measured as (%) (mean ± SEM) of worker honey bees exposed to an untreated control or ethion (79.47 mg a.i.L^{-1}), and hexaflumuron (500 mg a.i.L^{-1}) concentrations. The response of worker honey bees to the stimulus (CS and US) was analyzed by Chi-Square ($\chi 2$) tests. Different letters denote a significant difference between groups (A). The memory test occurred one hour after acquisition (B) ($p < 0.05$).

observed.

Water and sucrose consumption

Oral exposure to 50 % sucrose solution containing a sub-lethal concentration of ethion (47.79 mg a.i.L^{-1}) (F = 12.24, df = 4, P = 0.44) (Fig. 4A) and hexaflumuron in the field-recommended concentration (500 mg a.i.L^{-1}) (F = 0.65, df = 4, P = 0.16) (Fig. 4A) induced no effect on water consumption.

Oral ethion (47.79 mg a.i.L^{-1}) (F = 0.03, df = 4, P = 0.04) (Fig. 4B) induced a decrease in honeybees' sucrose consumption, but hexaflumuron (500 mg a.i.L^{-1}) (F = 1.00, df = 4, P = 0.13) did not affect honeybees' sucrose consumption (Fig. 4B).

Discussion

This study investigates the side effects of exposure to organophosphorus and IGR insecticides on learning, memory, water, sucrose consumption, and responsiveness in honey bees in Iran. Ethion and hexaflumuron are two widely used insecticides in agricultural systems in Iran (Talebi-Jahromi, 2011). Ethion is used on aphids, thrips, and scale insects of fruit and citrus trees (Talebi-Jahromi, 2011). Hexaflumuron has a good effect on the psylla of fruit trees (Shabani et al., 2011).

In the insect central nervous system, nerve impulse transmission is carried out by acetylcholine, Ach, and its hydrolyzing enzyme called acetylcholinesterase, AChE. The inhibition of acetylcholinesterase, AChE, activity in insects is the mode of action of organophosphorus insecticides (Talebi-Jahromi, 2011). Benzoyl urea (IGR compounds) interferes with the biosynthesis of chitin and leads to abnormal molting in insects (Talebi-Jahromi, 2011). Insecticides can exert not only lethal effects but also sub-lethal effects on insects. The sub-lethal toxicity of insecticides can be on non-target sites. For example, it is found that ultrastructural changes in target and non-target organs, such as Malpighian tubule and digestive cells, have been caused by honey bee exposure to thiamethoxam. consequently, reduce the health of honey bees (Friol et al., 2017).

The ethion and hexaflumuron concentrations chosen for this study were sub-lethal (47.79 mg a.i.L^{-1}) and field-recommended (500 mg a.i. L^{-1}) respectively (Delkash-Roudsari et al., 2022). In our experiments, we simulated the situation where honey bees are exposed to pesticides in food (sucrose solution). Most of the studies on the effects of pesticides on honey bee learning and memory are related to bee olfactory learning and memory, and few studies have been done on the effect of agricultural pesticides on bee visual learning and memory. Visual learning is an important ability for bees as it assists in finding the location of the hive, and in finding food sources (Lichtenstein et al., 2019). We found that ethion decreased visual learning and memory in honey bees, while hexaflumuron did not affect visual learning and memory.

Previous research showed that neonicotinoid pesticides such as thiamethoxam and imidacloprid disrupted honey bee visual learning (Ludicke and Nieh, 2020b; Han et al., 2010). Muth et al. (2019) for example, reported that the visual learning of bumblebees was not

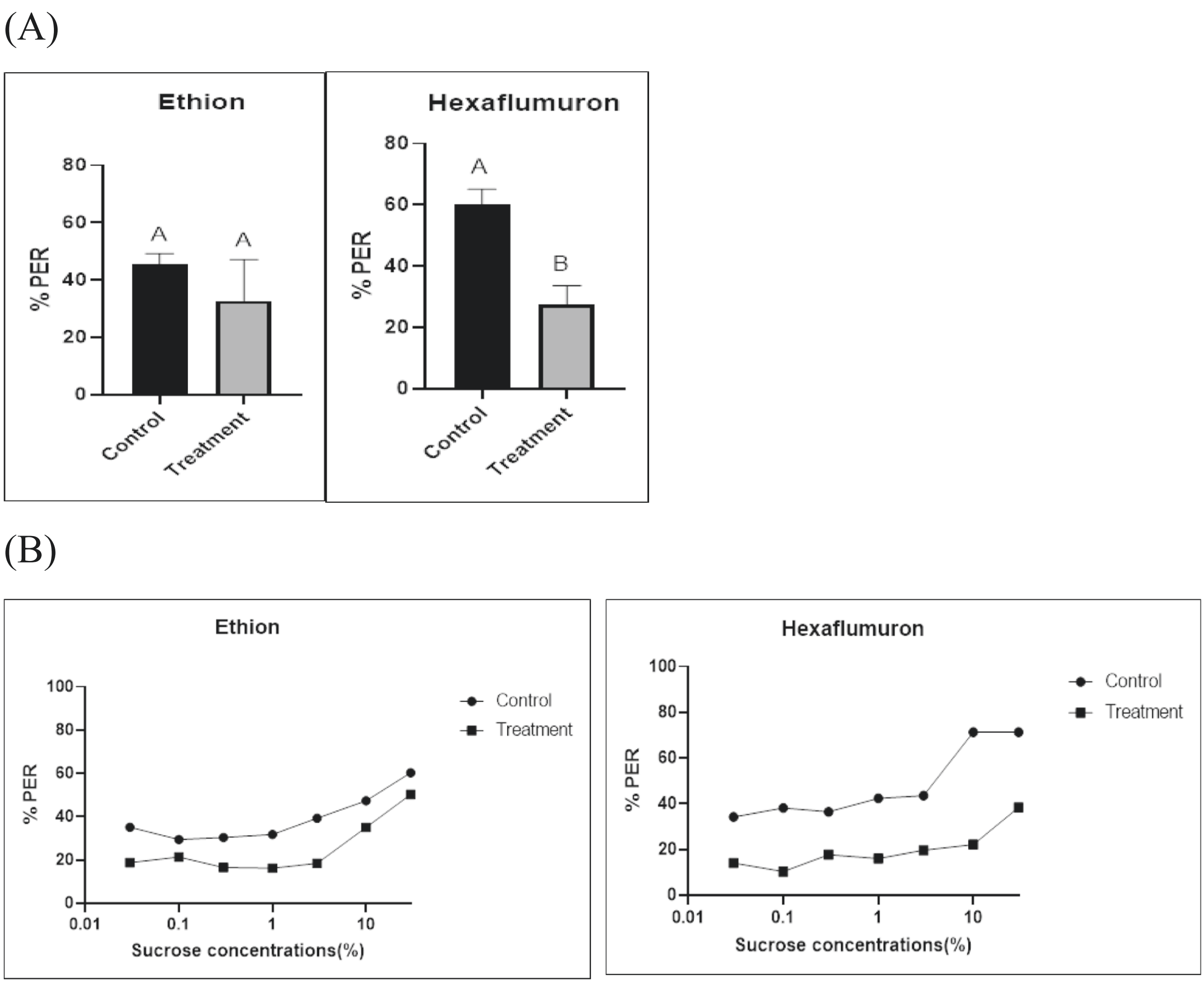

Fig. 3. Water (A) and sucrose responsiveness (B) measured as (%) (mean ± SEM) of worker honey bees exposed to an untreated control or ethion (79.47 mg a.i.L^{-1}), and hexaflumuron (500 mg a.i.L^{-1}) concentrations. Statistical analysis for sensory perception was performed by comparisons of the means using a T-test. Different letters denote a significant difference between groups (A). ($P < 0.05$).

impaired by thiamethoxam, imidacloprid, or clothianidin. It is also known that the acaricides thymol (Apiguard®) and fluvalinate (Apistan®) and the insecticide imidacloprid in field-relevant doses have no effect on visual learning and memory but the combination of thymol and imidacloprid reduced visual learning and memory performance (Colin et al., 2020). Another study found that the external treatment of honey bee colonies by acaricides amitraz and fluvalinate harms reproductivity, and honey productivity, and, probably, affects learning and memory, gustation, and olfaction (Ilyasov et al., 2021). Mancini et al. (2018) demonstrated that biological amines, which are chemical messengers in the nervous system have no negative effects on the visual learning and memory of honey bees.

Oral treatments of ethion and hexaflumuron significantly affected honey bee olfactory learning and memory. As evident from our results, ethion-treated honey bees exhibited a significant reduction in PER conditioning. This suggests a failure to learn the odor-reward association, indicating that honey bees did not successfully associate the two stimuli, and were unable to recall this connection afterward. Furthermore, the used organophosphate insecticide (ethion) in sub-lethal concentration was able to show its negative effect in the first trial of learning, especially on olfactory learning that is used by individuals both within and outside the hive.

Studies have shown that pesticides from different groups can have various effects on olfactory learning and memory in honey bees at certain concentrations (Abramson et al., 2004; Abramson et al., 1999; Frost et al., 2013; Al Naggar et al., 2015; Farina et al., 2019; Li et al., 2019). The concentration of insecticides used is a critical factor that can significantly influence the results of an experiment. Even two insecticides with the same mode of action can have different effects. For instance, Jason and Thorn (2002) demonstrated that coumaphos and diazinon (organophosphate insecticides) at an acute sub-lethal dose exhibited different effects on acetylcholinesterase activity, which, in turn, influenced olfactory learning.

Olfactory learning is important in foraging because it allows the honey bees to associate the nectar of flowers and odor. This ability helps bees find food resources. Flowers containing nectar can be identified by olfactory cues (Wright et al., 2009). The disruption of this ability may have serious consequences for colony fitness. As mentioned above, various factors can not only disrupt visual abilities but also reduce olfactory learning and memory capacities. Furthermore, it can be argued that rather than learning, the honey bees' motor systems are influenced by insecticides (Abramson et al.,1999).

Sucrose perception has an important effect on behavioral decisions made by honey bees. For instance, honey bees do different dance behaviors related to the sucrose concentrations offered by food resources. The performance of learning in honey bees is extremely influenced by sucrose response thresholds and the perception of sucrose concentrations used (von Frisch, 1965; Raveret-Richter and Waddington, 1993;

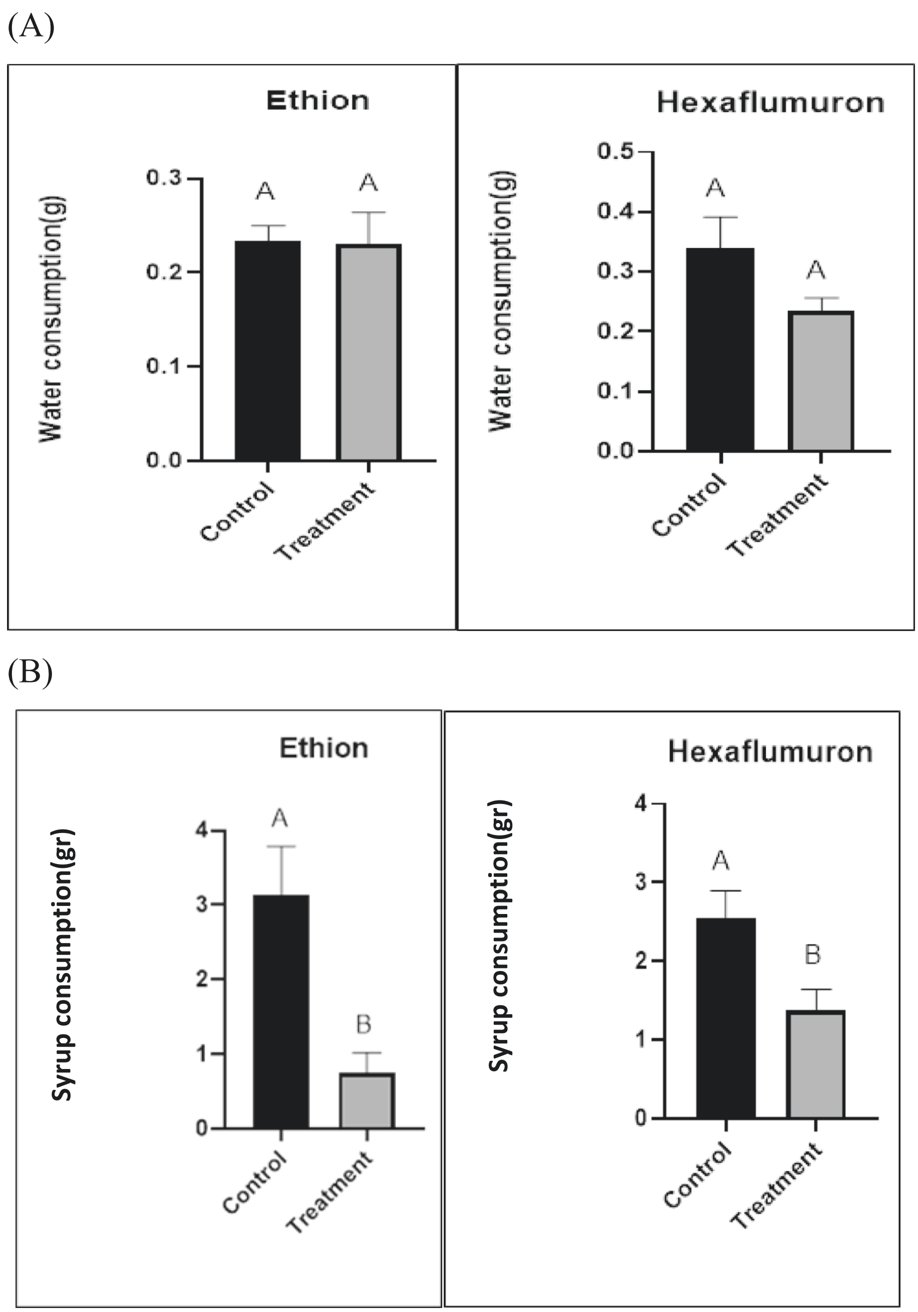

Fig. 4. Water (A) and sucrose consumption (B) measured as (%) (mean ± SEM) of worker honey bees exposed to an untreated control or ethion (79.47 mg a.i.L^{-1}), and hexaflumuron (mg a.i.L^{-1}) concentrations. Statistical analysis for sensory perception was performed by comparisons of the means using a T-test. $P < 0.05$.

Scheiner et al., 1999; Scheiner et al., 2001).

The results of our experiments show that honey bee ingestion of ethion reduced sucrose consumption and responsiveness, and hexaflumuron has a negative effect on water and sucrose responsiveness. These results illustrate that, despite the different modes of action for these insecticides, they can have adverse impacts on bees, which can ultimately endanger the health of the colony.

Previous work on water and sucrose responsiveness and consumption in honey bees has shown that imidacloprid, glyphosate, and acetamiprid had a negative effect on the response of bees to sucrose (El Hassani et al., 2008; Mengoni Goñalons and Farina, 2018). In contrast, Decourtye et al. (2005) and Bell et al. (2020) found that dimethoate had no adverse effects on sucrose responsiveness. Insecticide exposure can also have a negative effect on water and sucrose consumption (Aliouane et al., 2009; Wu et al., 2017). More generally, decreased responsiveness can be related to some antifeedant effects of insecticides. For example, Suchail et al. (2000) found that honey bees exposed to imidacloprid reduced trophallaxis.

The target site of organophosphates is acetylcholinesterase enzymes, which play a key role in clearing acetylcholine from its receptors in nerve cells such as ethion (Russell et al., 2004). Since acetylcholinesterase (AChE) is present in different parts of the insect's body (Thany et al., 2010) inhibition of AChE by organophosphorus insecticides leads to systemic disruption (Glavan and Bozic, 2013). The disruption can occur in the mushroom bodies where AChE is expressed and plays a role in learning and memory, (Heisenberg, 2003; Zars, 2000; Heisenberg, 2003; Belzunces et al., 2012; Blacquiere et al., 2012).

Insecticides may also influence signaling pathways, a series of chemical reactions in which molecules work together to control cell function. For example, it has been reported that fluvalinate impairs olfactory responses in honey bees. However, fluvalinate did not affect the expression of olfactory-related genes. Instead, it negatively affected the signaling pathways of small neuropeptide F (sNPF) related to olfactory responses (Lim et al., 2020). Moreover, Elizabeth Deeter et al. (2023) reported that spirodiclofen (an IGR insecticide) affects honey bee behavior by disrupting lipid homeostasis.

Conclusions

Overall, some essential findings emerge from our research: Pesticides can have a negative effect on the behavioral characteristics of bees in the field-recommended concentration and in the sub-lethal concentrations. Our results support the hypothesis that ethion and hexaflumuron have various side effects on honey bees. Further studies are needed to understand how insecticides can cause these different side effects. To determine the negative effects of insecticides, it is essential to investigate various behavioral characteristics rather than limiting an investigation to one characteristic. For example, we demonstrated that an insecticide such as hexaflumuron may impair visual learning without relevantly harming olfactory learning.

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.aspen.2024.102202.

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