

Non-target toxicity of novel insecticides

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Humans have used insecticides since ancient times. The spectrum and potency of available insecticidal substances has greatly expanded since the industrial revolution, resulting in widespread use and unforeseen levels of synthetic chemicals in the environment. Concerns about the toxic effects of these new chemicals on non-target species became public soon after their appearance, which eventually led to the restrictions of use. At the same time, new, more environmentally-friendly insecticides have been developed, based on naturally occurring chemicals, such as pyrethroids (derivatives of pyrethrin), neonicotinoids (derivatives of nicotine), and insecticides based on the neem tree vegetable oil (*Azadirachta indica*), predominantly azadirachtin. Although these new substances are more selective toward pest insects, they can still target other organisms. Neonicotinoids, for example, have been implicated in the decline of the bee population worldwide. This review summarises recent literature published on non-target toxicity of neonicotinoids, pyrethroids, and neem-based insecticidal substances, with a special emphasis on neonicotinoid toxicity in honeybees. We also touch upon the effects of pesticide combinations and documented human exposure to these substances.

KEY WORDS: *neonicotinoids; pyrethroids; neem; azadirachtin; non-target toxicity*

Since ancient times, humans have used insecticides to mitigate shortages in harvest potency (1). A major group of chemicals, broadly named *pesticides*, were introduced to agricultural practice exactly with the purpose to increase crop yield (2, 3). With time, their use has expanded to treating infestations in small animals (4), eradicating household insects (5), or preventing vector-borne infectious disease (6). With the beginning of industrial revolution came newly synthesised organic pesticidal compounds (7), whose widespread use soon increased the burden of synthetic chemicals on the environment. New problems began to arise, such as target species resistance (8) and non-target species toxicity (9, 10). Concerns about the adverse environmental impacts were aptly voiced in the book *Silent Spring* in 1962 (11). The first to be banned because of adverse effects on human health was DDT (12). This triggered a series of toxicological evaluations, many of which resulted in use restrictions or bans of environmentally excessively toxic and persistent substances.

Sustainable agriculture implies a set of practices to meet food and textile needs of the continuously growing world population without compromising the same need for future generations (13). One of these practices is the development and usage of new plant protection products based on naturally present compounds which protect plants against various pests and parasitic organisms. The pesticides that meet these requirements are referred to as “green” pesticides. They are expected to be effective, safe for non-

target organisms, and biodegradable in the environment (14). Pyrethroids and neonicotinoids are two showcase groups of the new pesticides that meet those needs. They are more selective for pest insects, less toxic to mammals, and biodegrade better than synthetic pesticides, yet, ever since the report implying them in bee population declines and other adverse effects on non-target organisms they have come under public scrutiny (15, 16).

Another natural pesticide, neem, has been used in traditional medicine in India thanks to the alleged antimicrobial and antiparasitic effects of its oil (17). In addition, neem extracts have been known to possess insecticidal properties, the most potent among them being azadirachtin (18).

This review aims to provide an updated overview of non-target toxic effects of these three groups of insecticides.

NEONICOTINOIDS

Nicotine, a well-known extract of *Nicotiana tabacum*, has been used as aphicide for centuries. However, its insecticidal potency is low and narrow, whereas its acute toxicity to mammals is high (19). To overcome these limitations, pesticide research developed compounds which have become known as neonicotinoids. They act as nicotine acetylcholine receptor (nAChR) agonists to prolong insect neuron depolarisation and overstimulate their central nervous system, which eventually kills them. Many of them are intended for preemergent use. Considering their systemic nature, neonicotinoids spread into all the tissues of the emerging plant. Representative substances of this

group are imidacloprid, acetamiprid, thiametoxam, thiacloprid, clothianidin, and dinotefuran.

Due to high selectivity for insect nAChRs, their acute toxicity is relatively low to mammals but is much higher if chronic.

Effects on non-target insects of economic importance

Honeybees

Honeybees are pollinator insects of great economic and ecological importance, but are susceptible to sublethal exposure to neonicotinoids. In 2006, honeybee keepers in the USA reported strange, inexplicable disappearances of honeybee drones from colonies, an event later named as “colony collapse disorder” (20). Since then, neonicotinoid insecticides were implicated as major culprits for the global honeybee and bumblebee population declines. Their systemic distribution throughout a plant, flowers and pollen in particular, poses a specific risk for pollinator insects (21). The risk of exposure also comes from consuming water polluted with neonicotinoids (22). Imidacloprid, acetamiprid, and clothianidin are very persistent water pollutants detected in biologically relevant concentrations even in treated wastewater (23). Honeybees could also be greatly exposed to neonicotinoid-containing particulate emissions stirred by drilling machines used in seeding (24).

The effects seem to vary by neonicotinoid type. In one study imidacloprid caused higher mortality in honeybees than acetamiprid (25). Thiacloprid, in turn, seems to impair their foraging behaviour, homing success, navigation performance, and social communication (16). Honeybee workers treated orally with sugar water containing imidacloprid delayed their return to the hives significantly (26). One meta-analysis (27) suggests that imidacloprid in nectar at field-realistic levels will have no lethal effects but will reduce the expected performance in honeybees by 6-20%. Another study (28) reported that their overwintering success depended on the dose of imidacloprid exposure through pollen. Another, Canadian study reported increased worker mortality and queenlessness in colonies near corn fields treated with neonicotinoids (29). In another study (30), oral exposure to imidacloprid subtly affected honeybee motor function. Teeters et al. (31) recorded reduced flight envelope and interactions in their study using a video. *In vitro* research also confirmed adverse effects. In cultured Kenyon cells from the honeybee brain, imidacloprid and clothianidin at concentrations seen in foraging honeybees and hives caused a depolarisation block and inhibited nicotinic responses (32). Neonicotinoid exposure can also affect bee short-term memory and associative learning, as demonstrated by Zhang et al. (33). In another study (34), young adult honeybees exposed to imidacloprid both orally and topically showed changes in gustatory responsiveness and learning and memory impairment, which may negatively reflect on the bee hives. Significant adverse

effects on learning performance were also established with a proboscis extension response (PER) assay in semi-field and laboratory conditions (35). The same study also found that syrup contaminated with imidacloprid decreased the foraging activity and the activity at the hive entrance. The forager return rate declined linearly with the increasing imidacloprid dose. El Hassani et al. (36), in turn, reported that acetamiprid increased antennal sensitivity to stimulation with sucrose solutions and impaired long-term retention of olfactory learning, whereas thoracic application induced no such effects but increased locomotor activity and water-induced PER.

Most of these studies are focused on oral or thoracic topical exposure to neonicotinoids, but one study (37) through increased mortality showed that wings are another relevant route of exposure and proved that significant amounts of pesticides could be delivered to the wings by air displacement during flight.

A metabolic study (38) quantifying imidacloprid and its metabolites 5-hydroxyimidacloprid and olefin in honeybee concluded that imidacloprid was responsible for immediate neurotoxicity symptoms, whereas its metabolites must have been responsible for mortality, since it occurred post-ingestion at which time no imidacloprid was detected. However, neither of the two studied metabolites were involved, which suggests that other metabolites were responsible for the mortality. Zhu et al. (39) fed honeybees with a commercial formulation of imidacloprid in sucrose solution and noted higher cytochrome P450 activity. According to another study (40), honeybees mainly rely on cytochrome P450 monooxygenases to counter the toxic effects of this imidacloprid formulation, but other authors claim that every active substance is countered by different enzymes, at least in bumblebees (41).

Honeybees fed imidacloprid and clothianidin via foraging on conventionally grown maize showed elevated acetylcholinesterase (AChE) activity (42). Changes in honeybee thorax temperature might be linked to impaired foraging and in-hive performance, as Tosi et al. (43) found both significant increases and decreases in honeybee thorax temperatures, depending on environmental temperature. They also performed a cold shock experiment, where two higher doses elicited a decrease, and a lower dose an increase in thorax temperature. Histology revealed significant alterations across several tissues, and one of the most common findings was apoptosis. In another study (44) the TUNEL assay, immunofluorescence, and real-time polymerase chain reaction (RT-PCR) revealed increased neuronal apoptosis and apoptotic markers caspase-3 and caspase-1 mRNA in honeybee brains, with concurrent autophagy. De Almeida Rossi et al. (45) treated honeybees with sublethal doses of imidacloprid and demonstrated cytotoxicity via Feulgen reaction, xyloidine ponceau staining, and immunocytochemistry, with optic lobes being the most sensitive honeybee brain region to imidacloprid exposure.

Oliveira et al. (46) also reported stronger xyloidine ponceau staining in the optical lobes of bees exposed to thiametoxam compared to control, whereas the cells taken from the midgut showed morphological and histochemical changes. Catae et al. (47) established toxic effects of thiametoxam on malpighian tubules, with organ damage increasing with exposure duration. Similar cytotoxicity in malpighian tubules was established for sublethal imidacloprid (48). The suggested mechanism of toxicity in honeybees is the inhibition of mitochondrial activity and depletion of adenosine triphosphate (ATP) (49). In one study (50), bumblebees exposed to imidacloprid showed reduced ATP and visual impairment, indicating a toxicity mechanism in common with honeybees.

According to Wessler and Kirkpatrick (51), neonicotinoids and other neurotoxic pesticides might also affect the non-neuronal acetylcholine (ACh) system (a term referring to cells using paracrine secretion of ACh to communicate outside the cholinergic nervous system), which seems to play a role in honeybee reproduction. Their action can therefore impair reproduction and limit biodiversity. Straub et al. (52) studied sexual maturation of bee drones from hives exposed to clothianidin and thiametoxam and found lower sperm viability and count of living sperms, as well as shorter drone lifespan. Gajger et al. (53) studied the reproductive morphology of honeybee queens and found lower sperm count in the spermathecae that received the higher thiametoxam dose. Chaimanee (54) reported lower sperm viability in queen honeybees exposed to imidacloprid.

Neonicotinoid exposure has also been shown to affect honeybee immunocompetence. For instance, Brandt et al. (15) have shown that exposure to field-realistic concentrations of thiacloprid and imidacloprid and to higher concentrations of clothianidin reduced haemocyte density, encapsulation response, and antimicrobial activity. In another study (55) clothianidin and imidacloprid induced immunosuppression via increased Nf-kB inhibition. In addition to honey production and total number of bees, imidacloprid significantly affected the activity of the immune-related enzyme phenoloxidase in forager bee extracts (56).

The effects of neonicotinoids on larval development have also been assessed. Tavares et al. (57, 58) reported lower larval and pupal survival and adult honeybee emergence for thiametoxam, as well as changes in acetylcholinesterase (AChE), glutathione-S-transferase (GST), carboxyesterase para, and alkaline phosphatase (ALP). In another study (59) newly emerged bees exposed to thiametoxam during the larval stage showed morphological changes suggestive of tissue degeneration in the digestive system, malpighian tubules, and Kenyon cells, likely to compromise their life span. Peng et al. (60) reported lower synaptic density in the calyx, which is responsible for the olfactory and visual functions, due to sublethal imidacloprid exposure of larvae. Yang et al. (61) studied the relation

between larval imidacloprid exposure and delays in larval development stages and found no effect at sublethal concentrations. However, the associative olfactory behaviour of adult bees was significantly impaired.

At the genetic level, Wu et al. (62) reported a strong downregulation of the genes encoding major royal jelly proteins (MRJPs) in newly emerged adults exposed to imidacloprid as larvae. This group of proteins is important for the sustainable development of bee colonies. Two other studies (63, 64) reported that sublethal thiametoxam may target genes linked to many functions, including behaviour, immunity, metabolism, biosynthesis, translation, and neural function, whereas another study (65) found a drop in vitellogenin, an egg yolk precursor that regulates honeybee development and behaviour.

Laboratory and field studies disagree about the extent of neonicotinoid exposure effects. Honeybees exposed to imidacloprid in laboratory cages showed generalised immunosuppression and the triggering of detoxification enzymes, whereas field-treated bees were more resilient (66).

Bumblebees

Bumblebees are another important pollinator species. They seem to suffer the adverse insecticide effects even at concentrations regarded as safe (67). Studies of bumblebee exposure to insecticides consistently show greater sensitivity of these species compared to honeybees. The difference in sensitivity between honeybees and bumblebees was explained by better honeybee adaptation to feeding on nectars containing synthetic alkaloids such as neonicotinoids by virtue of their ancestral adaptation to tropical nectars in which natural alkaloids are prevalent (68). Bumblebees have been reported to retain higher levels of imidacloprid after ingestion than honeybees (69).

Like in honeybees, neonicotinoids seem to diminish the foraging ability and homing success of bumblebees (70). Laycock et al. (71) reported lower survival, brood size, and feeding in bumblebees exposed to thiametoxam applied at 39 and 88 $\mu\text{g kg}^{-1}$ bw, but not at doses between 1 and 11 $\mu\text{g kg}^{-1}$ bw as found in nectars. According to Bryden et al. (72), sublethal exposure leads to initial growth and then reduction in bumblebee colonies. However, different species of the *Bombus* genus are not equally sensitive to sublethal insecticide effects. Baron et al. (73) exposed wild queens of four bumblebee species [*B. terrestris* (Linnaeus), *B. lucorum* (Linnaeus), *B. pascuorum* (Scopoli), and *B. pratorum* (Linnaeus)] to thiametoxam and found reduced feeding in two species.

Neonicotinoids also affect the immune system of bumblebees. Imidacloprid lowered the activities of phenoloxidase and hemolymph and reduced bumblebee survival following non-pathogenic immune challenge (74).

As for the neurological effects, according to an *in vitro* study (75), imidacloprid and clothianidin can affect

bumblebee behaviour by stimulating neural Kenyon cells. In another study (76), bumblebees chronically exposed to field-realistic levels of thiametoxam learnt more slowly, and their short-term memory was significantly impaired. Experiments also show that successive generations of bumblebees adapt to imidacloprid exposure. In one study (77) neural cells of the newly emerged adult workers were less sensitive to imidacloprid exposure than the neural cells from older workers.

Different substances of the same class tend to have different effects on bumblebee cells, individuals, and whole colonies. In a study by Moffat et al. (75), imidacloprid and thiamethoxam, but not clothianidin, affected bumblebee colony strength, whereas thiametoxam was the only substance that affected the sex ratio (in favour of males).

Two studies even reported recovery from exposure to imidacloprid (69, 78). Acetamiprid has consistently been reported as the least toxic neonicotinoid. It first entered the European market following the approval by the European Food Safety Authority (EFSA) (79) due to its low non-target toxicity and low potential for bioaccumulation. Further studies supported its low toxicity to honeybees (36, 80,-81). Acetamiprid may owe its low toxicity to its chemical structure, which is different from older neonicotinoids such as imidacloprid. Acetamiprid is a cyano-substituted and imidacloprid a nitro-substituted compound (82). Furthermore, acetamiprid seems to be the least likely to enter a beehive (83).

Just as for honeybees, laboratory and field studies disagree about the extent of neonicotinoid exposure effects in bumblebees. Arce et al. (84) tried to bridge this disagreement by exposing bumblebees to controlled doses of clothianidin in sucrose syrup and by assessing their foraging activities and the number of workers and sexuals. The effect on foraging was small, but the number of bees dropped significantly.

Combined effects of neonicotinoids, parasites, and other stresses on pollinators

Since pollinators are rarely exposed to a single pesticide, many studies examined the combined effects of pesticides on non-target species. Liu et al. (85) exposed honeybees orally for 48 h to clothianidin, thiametoxam, and dinotefuran separately and in binary and ternary combinations. All combinations had additive or synergistic toxic effects.

Parasite infestations also seem to worsen the adverse effects of neonicotinoid pesticides, and the combination seems to be one of the main reasons for global pollinator decline (86, 87). In combination with infectious microspores, however, it increased short-term mortality and led to long-term colony decline (88). Imidacloprid exposure also reduced flight capacity of honeybees affected by the mite *Varroa destructor* (Anderson & Trueman) (89). In an observational study in the Netherlands, *V. destructor* mite infestation and the presence of acetamiprid and thiacloprid

were the two most significant factors in winter honeybee losses (90). Sublethal doses of thiacloprid caused higher mortality in honeybees infected with *Nosema ceranae* (Fries) than in the uninfected ones (91). Field-realistic nutritional stress and pesticide exposure were also reported to synergistically diminish honeybee survival (92). All these findings point to a greater susceptibility to pathogens in neonicotinoid-exposed pollinators (93).

Another stressor contributing to bee population decline is poor diet, whose effects are even worse if combined with a neonicotinoid, as reported by Dance et al. (94) for bumblebees feeding on monofloral pollen coated with thiametoxam.

Non-target toxicity of neonicotinoids in other insects and invertebrates

Apis cerana (Fabricius) is another aphid species prevalent in Asia. Because of its smaller mass, it would be reasonable to assume that this species is more sensitive to neonicotinoids than *Apis mellifera* (Linnaeus), but Yue et al. (95) reported that both species were equally sensitive to dinotefuran, *A. cerana* (Fabricius) was more sensitive to acetamiprid, and *A. mellifera* (Linnaeus) was more sensitive to imidacloprid and thiametoxam. One study with a stingless bee species *Osmia cornuta* (Latreille) found impaired visual guidance and navigational memory caused by clothianidin (96). Whitehorn et al. (97) found an interesting effect of imidacloprid on *Nasonia vitripennis*, as it produced more females in the offspring than control. Studying ants, Thiel et al. (98) noted delays in the recruitment of new workers in the *Lasius niger* (Linnaeus) species and fewer foragers and greater aggression in the *L. flavus* species. Another study found lower interspecies aggression and higher survival in a native species and higher interspecies aggression and lower survival in the invasive species, both exposed to imidacloprid (99).

Trophic transfer has been proposed as an important route of entry for neonicotinoids into the ecosystem, as thiametoxam from treated soybean travelled through herbivorous slug species into a predatory arthropod in the amounts sufficient to reduce its population density (100). This is another, albeit under-researched way insecticides can harm beneficial insects and disrupt biological pest control.

Cavallaro et al. (101) reported lower toxicity of thiametoxam in *Chironomus dilutus* than that of imidacloprid and clothianidin yet it does not make it environmentally less dangerous, as it quite likely degrades into clothianidin in the environment.

In *Chironomus riparium* (Meigen) low concentrations of thiametoxam inhibited growth, catalase activity, and caused lipid peroxidation (102). Azevedo-Pereira et al. (103) reported an imidacloprid-related drop in AChE activity, which correlated with reduced locomotion and ventilation in the same species.

Kobashi et al. (104) tested the survival of several aquatic insect species exposed to imidacloprid and dinotefuran and noted that it dropped in *C. servilia mariannae* (Drury), *L. pachygastra* (Selys), *N. triguttata*, and *G. japonicus* exposed to imidacloprid, but not to dinotefuran.

Malev et al. (105) exposed a crustacean amphipod *Gammarus fossarum* (Koch) and freshwater algae *Desmodesmus subspicatus* (Chodat) to imidacloprid active substance, its commercial formulation, and to its metabolite, 6-chloronicotinic acid and found reduced algal growth and elevated catalase activity and lipid peroxidation. Ugurlu et al. (106) found vacuolisation and haemostatic infiltration in the gill cells of *Gammarus kischineffensis* (Schellenberg) exposed to sublethal concentrations of thiametoxam.

Arican et al. (107) found reduced feeding in copepods exposed to thiacloprid and proposed feeding response as a more sensitive endpoint for ecological risk assessment than acute or chronic toxicity.

Imidacloprid was also shown to be toxic to earthworm *Eisenia fetida* (Savigny) (108, 109) and *Hyalella azteca* (Saussure) (110). *Eisenia andrei* (Bouché) showed bioaccumulation of various pesticides, which correlated with DNA damage (111). Van Hoesel et al. (112) also found reduced surface activity of earthworms in pots with wheat treated by seed dressing formulation based on imidacloprid and a fungicide prothioconazole.

Neonicotinoid effects on vertebrates

Early developmental exposure to imidacloprid has persisting effects on neurobehavioral function in zebrafish (113). Both active imidacloprid and its commercial formulation accumulated in the gut, gills, liver, and muscles of *Australoherus facetus* (Jenyns) and had genotoxic effects (114). Ansoar-Rodriguez et al. (115) found histopathological changes and increased levels of heat-shock-protein (Hsp) 70, pointing to hepatotoxicity in *Oreochromis niloticus* (Linnaeus). Xia et al. (116) reported genotoxicity, a decrease in hepatic enzymes glutamic-pyruvic transaminase and glutamic-oxalacetic transaminase, and histological changes in the testes of adult pond loach [*Misgurnus anguillicaudatus*, (Cantor)] exposed to sublethal concentrations of imidacloprid.

Imidacloprid exposure of chick embryos could increase the risk of neural tube defects and dysplasia (117). Lopez-Antia et al. (118) exposed *Alectoris rufa* partridges (Linnaeus) to imidacloprid-coated seeds and found increased mortality at recommended application concentrations, reduction in clutch sizes and egg laying delays, as well as reduced T-cell immune response. Imidacloprid was also reported to impact bobwhite quail [*Colinus virginianus* (Linnaeus)] embryonic development and chick survival, but this depended on specific exposure windows (119). Developmental delay was also noted in chick embryos (120, 121) exposed to imidacloprid, as well as histological changes in the chick cerebellum (122).

Male C57BL/6N mice exposed to a single dose of no-observed-adverse-effect level (NOAEL) of clothianidin showed elevated anxiety and increase in c-fos immunoreactivity in certain brain regions (123). Imidacloprid was also reported to stimulate high-fat-diet-induced adiposity and insulin resistance in mice (124). Male mice exposed to 1 mg kg⁻¹ bw of imidacloprid *in utero* and during lactation showed elevated aggressive and sexual behaviour, suggesting male-specific impairment of neural development (125). Exposure of gestating mice to clothianidin also reduced germ cell count in the offspring without hormonal changes (126). Still in mice, Hirano et al. (127) reported clothianidin-induced behavioural and reproductive effects, which were more pronounced under chronic stress. Treatment of mouse embryos with 1000 µmol L⁻¹ of thiametoxam, clothianidin, acetamiprid, and thiacloprid during the preimplantation period had negative effects on embryo developmental abilities (128). Exposure to imidacloprid at concentrations higher than 10 µmol L⁻¹ for less than one minute changed the membrane properties of mouse stellate cells with AChRs (from the cochlear nucleus) and consequently their function (129). Ten-week-old mice drinking water spiked with acetamiprid at ten and hundred times the NOAEL had the substance concentrated in the midbrain region, and their body weight decreased (130).

In rats, high doses of acetamiprid reduced liver weight and changed haematological parameters and liver ALT, AST, ALP, and LDH activities (131). Rats exposed to high doses of thiametoxam for seven days showed increased anxiety and a drop in AChE activity, which points to the affected cholinergic system (132). 10 mmol L⁻¹ of thiametoxam and 2 mmol L⁻¹ of clothianidin increased extracellular dopamine levels when injected into the rat brain striatum, further characterising its mode of action in mammalian brain (133). Commercial thiacloprid, alone and in combination with deltamethrin, elevated free T3 and T4 hormone levels in rat serum in both single-dose and 30-day exposure (134). Subchronic (110 mg kg⁻¹ bw) administration of acetamiprid significantly decreased lymphocyte proliferation and macrophage function (135). Exposure to high doses of clothianidin (24 mg kg⁻¹ bw) affected the cognitive function of infant Wistar rats (136). At low doses clothianidin moderately affected the reproductive system of male rats exposed *in utero* (137). In contrast, a daily three-month exposure to imidacloprid (8 mg kg⁻¹ bw) significantly impaired the testicular function of adult male rats (138).

Hsiao et al. (139) reported impaired spatial memory in bats after chronic exposure to imidacloprid. They explained it with neuronal apoptosis in their brain.

Finally, dogs also seem to be affected by neonicotinoids. An epidemiological study by Gookin et al. (140) established an association between imidacloprid exposure and gallbladder mucocele formation, with the risk even higher in Shetland sheepdogs.

Human exposure to neonicotinoids

An observational study (141) noted a potential link between maternal exposure to agricultural pesticides such as neonicotinoids and pyrethroids and poorer neurodevelopment in children. A slight correlation between maternal exposure and autism spectrum disorder in children has also been reported by Keil et al. (142). According to a longitudinal study based on Texan poison centre reports, adverse events in people exposed to neonicotinoids were not severe and most of them were managed outside medical facilities (143).

Pyrethroids

Pyrethrins are naturally occurring compounds obtained by extraction from pyrethrum [*Chrysanthemum cinerariaefolium* (Linnaeus)]. They have insecticidal properties but are unstable when exposed to sunlight. Research into modifications of their structure has led to the development of synthetic pyrethroids with greater stability and insecticidal activity (19). These include cypermethrin, cyhalothrin, permethrin, bifenthrin, (es)fenvalerate, and cyfluthrin. Their mechanisms of action involve binding to voltage-gated sodium channels in insect neurons, preventing repolarisation, and inducing paralysis. Based on their structure, differences in specific electrophysiological effects, and poisoning syndromes in rats, they have been divided into type I and type II pyrethroids; the former causes aggressive behaviour, ataxia, convulsions, and progressive paralysis, whereas the latter causes choreoathetosis, salivation, and tremors (144).

Many of these substances have chiral carbon atoms that create enantiomers with identical physicochemical properties but different biological effects because of the binding stereoselectivity to receptors and other macromolecules within biological systems. This tends to complicate matters regarding their toxicology (145). Many studies treat different enantiomers in the racemic mixture as a single compound (146), yet their behaviour may differ. Yang and Ji (147) reported different degradation rates of the four beta-cypermethrin isomers they studied in soil. Likewise, enantiomers can differ in toxicokinetics. De Albuquerque et al. (148) reported that rat liver microsomes metabolised the (-)(1S-cis- α R) diastereoisomer of α -cypermethrin faster than the (+)(1R-cis- α S) diastereoisomer. They concluded that the stereoselective effects of chiral pesticides should be evaluated both in the environment and non-target species. Stereoselectivity was also demonstrated with DDT and cancer progression in MCF-7 cells (149). Chang et al. (150) reported different bioaccumulation effects on the thyroid hormone of two lambda-cyhalothrin enantiomers. Stereoselective toxicokinetics has also been reported for cycloxaprid in rats (151). All these reports show that it is very important to specify whether a racemate (such as cypermethrin), an

enriched enantiomer mixture (such as alpha-cypermethrin), or a pure enantiomer substance was used in toxicity studies.

Pyrethroid effects on non-target insects and invertebrates

Similar to neonicotinoids, pyrethroids have also been suspected to cause honeybee population declines. In one study (35) deltamethrin was lethal to worker bees and reduced their foraging activity at food source and hive entrance activity. In another study (152), cypermethrin inhibited Na/K ATPase in emerging honeybees in laboratory conditions. In caged honeybees it altered the expression of the genes involved in the immune system, cytochrome P450 enzymes, subunits of nAChRs, and vitellogenin (153).

Evidence of adverse effects abounds for other insects as well. Kuncce et al. (154) exposed damselfly larvae to deltamethrin and esfenvalerate and found reduced predatory ability with deltamethrin and GST inhibition with combined exposure. No effect of exposure to lambda cyhalothrin through biofilm on mayfly mortality has been reported, unlike direct exposure of mayflies through insecticide-spiked water (155). In yet another study (156), deltamethrin, lambda-cyhalothrin, and esfenvalerate were more toxic to *Iphiseiodes zugalai* (Denmark & Muma) larvae and adult mites than neonicotinoids imidacloprid and thiametoxam in terms of mortality, maturation, fecundity, longevity, and duration of adverse effects. Some insect species differ in sensitivity to pyrethroid exposure, as of the two predatory insects exposed to deltamethrin, *Buenoa tarsalis* (Truxal) was more sensitive than *Martarega Bentoi* (Truxal). (157)

In crustaceans, interactions between several pyrethroids tend to produce additive toxic effects, but some interactions were antagonistic, as reported for *H. azteca* (158). Alpha-cypermethrin showed adverse effects on *Daphnia magna* (Straus) adult growth and neonate size and number (159).

A combination of permethrin, lambda-cyhalothrin, and chlorpyrifos induced response in 12 of 15 zooplankton species, with the most sensitive species being the Radix sp. snail, *H. azteca*, *D. magna*, and copepods. *H. Azteca* and *D. magna* showed acute toxicity, and the snails and copepods a delayed sublethal response (160). One study (161) reported the prevalence of permethrin and cypermethrin in urban river sediment in South China, whose levels were toxic to *C. dilutus*.

Pyrethroid effects on non-target vertebrates

Pyrethroids have been shown to exert endocrine disruption in fish. In embryonic zebrafish, permethrin increased thyroxine and 3,5,3'-triiodothyronine levels and the transcription of most target genes involved in the hypothalamic-pituitary-thyroid axis (162). Simultaneous exposure to other insecticides can exacerbate symptoms of toxicity. Fai et al. (163) reported that *O. niloticus* was more sensitive to cypermethrin and deltamethrin than the organophosphate (OP) pesticide dimethoate, but together

the three showed synergistic toxicity, possibly due to OP inhibition of the pyrethroid-hydrolysing esterases.

In toads, deltamethrin caused oxidative stress, affecting the catalase, glutathione reductase, and GST activities in different tissues (164).

Mice fed with formulated cypermethrin for 60 days exhibited chromosome aberrations, micronuclei, reactive oxygen species, and disturbed cell cycle (165). Bardullas et al. (166) noted hypothermia in infant rats exposed to low doses of cypermethrin and bifenthrin, along with mild to moderate behavioural effects. Gamma-cyhalothrin, an enriched mixture of cyhalothrin isomers, was more neurotoxic to rats than lambda-cyhalothrin (167). Still in rats, a low-dose permethrin induced changes in liver histology, increase in lipid peroxidation, and a drop in superoxide dismutase activity in one study (168) and was responsible for altered *Nurr1* gene expression and reduced genome-wide DNA methylation in the offspring of pregnant rats in another (169). Upregulation of *Nurr1* was later associated with lower antioxidant concentrations (170). Pregnant dams treated with 10 mg kg⁻¹ bw of deltamethrin showed transient neurotoxicity and weight loss, but no effect on testosterone synthesis was observed in their male offspring (171). Beta-cyfluthrin and bifenthrin affected behaviour in adult and neonate rats, which may be related to a drop in AChE activity and the elevated oxidative stress in their brains (172). Another study also showed that the blood-brain barrier of infant rats was more permeable to deltamethrin than that of older rats (173). Allethrin, one of the first pyrethroids, decreased spermatogenesis and sperm function in male rats by downregulating the mRNA expression of factors important for testosterone levels, sperm count, and sperm function (174).

In mice, pulsed exposure to low-dose (3 mg kg⁻¹ bw) deltamethrin during gestation and lactation showed region-specific downregulation of Na-v mRNA and brain-derived neurotrophic factor, suggesting a mechanism for behavioural effects (175). Fenvalerate, known as endocrine disruptor from other studies, restricted intrauterine growth by disrupting placental thyroid hormone receptor signalling (176). In another study (177) cypermethrin applied at environmentally relevant doses to male mice significantly accelerated the onset of puberty by interfering with the hypothalamic sodium channels, pituitary gonadotrope calcium channels, and testicular Leydig cells, which all led to higher FSH, LH, and testosterone levels.

Exposure of male rabbits to two doses of cypermethrin for two and four months led to increased genotoxicity in the lymphocytes and cytotoxicity in the liver and kidney, as demonstrated by the cytokinesis-block micronucleus assay and histological examination (178).

Human exposure to pyrethroids

Many studies have assessed the links between residential and occupational exposure to pyrethroids and

potential health effects. Vidi et al. (179) reported a significant association between bifenthrin, cypermethrin, deltamethrin, *cis*- and *trans*-permethrin and DNA damage in the hair of residentially exposed children in farmer households. An observational study using data from poison control centres reported that the second most common pesticide group by exposure frequency were pyrethroids, and children aged ≤5 years were the group most at risk of exposure (180). Campos et al. (181) suggested an association between pyrethroid exposure and mental disorders. Poorer neurodevelopment in children in relation to maternal exposure to pyrethroids was further demonstrated by Gunier et al. (141). Furlong et al. (182) found a correlation between the levels of pyrethroid urinary metabolites 3-PBA and DCCA and behavioural deficits in children. Another study reported a stronger link between indoor pyrethroid exposure and autism spectrum disorder in the children of mothers who received less folic acid during pregnancy than those who received more (183). In a cross-sectional study of female farm workers in Tanzania, occupational exposure to pesticides affected haematological and biochemical parameters such as haematocrit, red cell corpuscular volume, serum glutamic oxaloacetate transaminase, and esterase (184). One case of pyrethroid skin necrosis was reported in a 67-year old diabetic woman suffering from delusions of parasitosis (185). In a recent review Martenies et al. (186) identified four studies showing an association between urinary metabolites of pyrethroids and lower semen concentration. Chiu et al. (187), however, reported the opposite: consumption of fruit and vegetables with low-to-moderate pesticide residues was associated with improved total sperm count and sperm concentration. The authors suggested that pesticide residues in fruit and vegetables could offset the beneficial effects of antioxidants and other naturally occurring chemicals in human organism. So far, however, there are too few studies of this type to infer anything more than that an association may exist. Similar to the finding in mice above (177), an observational study reported a positive correlation between pyrethroid urinary metabolites 3-PBA and 4-F-3PBA and gonadotropin levels (FSH and LH) and early-onset puberty in Chinese boys (188).

NEEM-BASED INSECTICIDES

Unlike the previous two groups of naturally occurring insecticides, little is known about the non-target toxicity of neem-based insecticides. What literature there is, it mainly reports no adverse environmental effects. However, one study reported azadirachtin suppression of otherwise beneficial phosphate-solubilising bacteria in soil (189). In another study, neem-based formulations changed the composition of zooplanktonic communities at higher concentrations (190). In adult copepods, azadirachtin reduced their population (191), which was also noted in

another study with adult copepods in pond enclosures (192). This adversely affects zooplankton food web stability. Similar perturbations in planktonic communities were also noted by Kreutzweiser et al. (193). Goktepe and Plhak (194) confirmed direct adverse effects on aquatic organisms with azadirachtin formulations more resistant to degradation by sunlight.

Regarding non-target insect effects, exposure of bumblebees to azadirachtin caused a variety of toxic effects, including reduced offspring mass, absence of reproduction, and mortality (195). Neem oil affected cocoon spinning in *Ceraeochrysa claveri* (Navás), possibly increasing their vulnerability to natural enemies and environmental factors (196).

There are several reports of neem-based compounds affecting fish. Intramuscular application of low-dose azadirachtin in *Oreochromis Mossambicus* (Peters) caused chromosomal aberrations (197). In carp it significantly altered locomotor and haematological parameters (198). In catfish it affected leukocyte populations, which pointed to a possible immunotoxicity (199). Azadirachtin also caused histological changes to ultimobranchial gland, an organ important for calcium homeostasis, in the Asian stinging catfish [*Heteropneustes fossilis* (Bloch)], but only with prolonged exposure (200). In zebrafish, it increased general activity and anxiety-like symptoms, but there was no effect on learning (201).

As for mammals, neem extract seems to be spermatotoxic in rats (202). In another study (203) high concentrations of azadirachtin reversibly affected rat neuron excitability by modulating potassium conductances. Vepacide, a neem oil-based preparation, increased aspartate and alanine aminotransferase in the serum, kidney, and lung, but decreased them in the liver of rats, suggesting liver necrosis (204). Subchronic exposure of Wistar rats to vepacide induced biochemical changes in the levels of acid and alkaline phosphatase (205). In contrast, azadirachtin showed no signs of toxicity in either gestational rats or their offspring (206), or in subchronically treated adult rats of either sex (207).

Little is still known about the risks for humans. What we know is that azadirachtin has no genotoxic effect on human cells, but may have antiproliferative effects, as evidenced by the changes in the cell cycle of human lymphocytes, some of which also displayed aberrant mitoses and poliploidy with multipolar spindles (208). One study reported that an azadirachtin-based nematicide caused eye irritation but no systemic effects (209). There is also a case report of a 35-year-old woman showing transient signs of neurotoxicity after ingesting azadirachtin in a suicide attempt (210).

CONCLUSIONS

Experimental evidence suggests diverse toxicities of the three new, presumably selective and environmentally safe insecticides in non-target, environmentally beneficial insects, planktonic organisms, fish, and mammals. With pyrethroids this is in no small part due to their stereoselectivity. Although an increasing number of studies now acknowledges stereoselectivity as an important factor in toxicological assessment, more research is needed on this subject.

The reviewed studies have also shown that insecticides when mixed, which corresponds to real-life exposure patterns, mostly interact to produce additive or synergistic effects, but antagonisms have also been noted. Further research should investigate the mechanisms of these interactions on the molecular level.

Many practical measures can be put to use to buffer the negative impacts of pesticides on the environment, such as rotating different insecticides (39), combining common insecticides with detoxification-enzyme-inducing pesticides (40), and avoiding pesticides affecting beneficial predatory arthropods used in biological pest control. The risk to pollinators and other non-target organisms may also be reduced by aligning the use of insecticides with pest incidence (211). Furthermore, treating certain species such as bumblebees with near infrared light seems a promising way to counter imidacloprid effects (50).

Promising improvements also come with even newer alternative insecticides, such as a spider-venom derivative atracotoxin that targets calcium channels. In an early-tier risk assessment (212) it did not affect honeybee mortality or learning, nor was it toxic to larvae. Of course, full risk assessment should involve the whole lifecycle of colony health and include other species and interactions with other stressors such as pathogens.

Regarding neem-based pesticides, further research into the effects on beneficial pollinator species is needed to complement its relatively favourable toxicological profile in mammals.

Conflict of interest

None to declare.

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Neciljana toksičnost novih insekticida

Ljudi su upotrebljavali insekticide od davnih vremena. Spektar i potentnost dostupnih insekticidnih supstancija značajno su se proširili od industrijske revolucije, vodeći širokoj upotrebi i neviđenim količinama sintetskih kemikalija u okolišu. Zbog toksičnih učinaka tih novih kemikalija na neciljane vrste javnost je ubrzo pokazala veliku zabrinutost, što je dovelo do ograničenja u korištenju tih insekticidnih supstancija. Istovremeno dolazi do razvoja novih metoda u održivoj poljoprivredi, među kojima i razvoj novih insekticida na osnovi prirodno prisutnih kemikalija, poput piretroida (derivati piretrina iz suncokreta) i neonikotinoida (derivati nikotina). Dodatni su primjer insekticidi na bazi biljnog ulja iz drveta nima (*Azadirachta indica*), dominantno azadiraktin. Iako su te nove supstancije selektivnije prema insektima, poteškoće nastaju s njihovom neciljanom toksičnošću, poput one neonikotinoida, koji su utjecali na pad populacije pčela na globalnoj razini. Ovaj pregled sažima nedavnu literaturu o neciljanoj toksičnosti neonikotinoida, piretroida i insekticidnih supstancija na bazi nima, s naglaskom na toksičnosti neonikotinoida za pčele. Također ćemo se dotaknuti učinaka mješavina pesticida, kao i zabilježene ljudske izloženosti tim supstancijama.

KLJUČNE RIJEČI: *neonikotinoidi; piretroidi; nim; azadiraktin; neciljana toksičnost*