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The synergy of xenobiotics in honey bee *Apis mellifera*: mechanisms and effects

Sinergizem ksenobiotikov v medonosni čebeli *Apis mellifera*: mehanizmi in učinki.

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Abstract: During foraging activities honeybees are frequently exposed to different xenobiotics, most of them are agrochemical pesticides and beehive chemicals. Many pesticides are applied together and synergism is likely to occur in different organisms. The risk of synergisms is neglected and relatively few studies were performed concerning the effects and synergy mechanism of different xenobiotic combinations in honeybees. The understanding of synergy mechanisms between xenobiotics is very important for the control of defined mixtures use and also for the prediction of potential toxicity of newly developed substances in agriculture and apiculture. This review is focused on the effects, mechanisms and molecular targets of xenobiotics in honeybees and possible complex mechanisms of their synergisms. The main threat for honeybees are insecticides which primary molecular targets are few neuronal molecules therefore causing the impairment of neuronal system that have a profound effect on honeybee behavior, cognitive functions and physiology. However, the majority of synergistic effects observed in honeybees were ascribed to the inhibition of detoxifying midgut enzymes P450 involved in xenobiotic metabolism since most of studies were done with the mixtures xenobiotic/P450 inhibitor. The main inhibitors of P450 enzymes are specific compounds used to prolong the effects of pesticides as well as some fungicides. Some insecticides can also interact with these enzymes and influence the xenobiotis. Although the primary mechanisms of action of individual xenobiotics especially insecticides are well known and there are possible interactions in honeybees at their primary target sites, this issue is underestimated and it warrants further investigation.

Keywords: synergism, xenobiotic, A*pis mellifera*, mechanism, pesticide, P450

Izvleček: Medonosne čebele so med iskanjem hrane pogosto izpostavljene različnim ksenobiotikom, večinoma so to fitofarmacevtska sredstva in panjske kemikalije. Številna fitofarmacevtska sredstva se uporablja skupaj in znano je, da lahko pride do sinergističnih interakcij v organizmih. Tveganje za nastanek sinergizmov je podcenjeno in narejenih je relativno malo študij na čebelah o učinkih in mehanizmih sinergizmov različnih kombinacij ksenobiotikov. Razumevanje mehanizmov sinergizmov ksenobiotikov je zelo pomembno za nadzor nad uporabo definiranih

mešanic in napovedovanje potencialne toksičnosti novih ksenobiotikov v kmetijstvu in čebelarstvu. Pregledni članek se osredotoča na učinke, mehanizme in molekulske tarče ksenobiotikov v medonosnih čebelah in osvetljuje morebitne primere ter mehanizme nastanka sinergizmov. Najbolj nevarni za čebele so insekticidi, katerih primarne tarče so nekatere molekule živčnih celic,zato le-ti motijo delovanje živčnega sistema. Insekticidi zato lahko močno vplivajo na vedenje, kognitivne funkcije in fiziologijo čebel. Kljub temu raziskovalci večino sinergijskih učinkov v čebelah razlagajo z inhibicijo črevesnih detoksifikacijskih encimov P450, ki presnavljajo ksenobiotike, saj je bila večina študij narejena z mešanicami ksenobiotik/zaviralec encimov P450. Glavni zaviralci encimov P450 so specifični inhibitorji za podaljšanje učinka fitofarmacevtskih sredstev ter nekateri fungicidi. Tudi nekateri insekticidi lahko vplivajo na delovanje encimov P450 in tako vplivajo na interakcije med ksenobiotiki. Čeprav so primarni mehanizmi delovanja posameznih ksenobiotikov, še posebej insekticidov, precej znani in so sinergizmina ciljnih tarčah pri čebelah možni, je to področje podcenjeno in neraziskano.

Ključne besede: sinergizem, ksenobiotik, *Apis mellifera*, mehanizem, pesticidi, P450

Introduction

In addition to gathering nectar to produce honey, honey bees carry out another crucial function: pollination of agricultural crops, home gardens, orchards and wildlife habitat. A substantial decline of honey bee populations so called colony collapse disorder was observed in the last 15 years in many countries in Europe and in North America (vanEngelsdorp and Meixner 2010).Colony numbers in Europe for example decreased from over 21 million in 1970 to about 15.5 million in 2007 (FAO, 2009),a severe decline occurred after 1990. Many factors such as diseases, parasites, xenobiotics (pesticides and veterinary products), the environment, and socio-economic factors probably influence managed bee population, working alone or in combinations (vanEngelsdorp and Meixner 2010).

Honey bees may frequently become exposed to xenobiotics, environmental chemicals as a consequence of their foraging activities. Most of them are agrochemical pesticides and beehive or veterinary products, many of them of insecticide action, used against parasitic honey bee mites: *Acarapis sp.*, *Varroa destructor* and *Aethina tumida* (Thompson 2012). The use of pesticides to control weeds, fungi and arthropod pests seems inevitable in modern agriculture which seeks for the highest yields of the produces. Nectar foraging bees are likely to experienced highest exposure to both sprayed and systemic seed and soil treatments compounds followed by nurse and brood-attending bees. The residues of pesticides were found in pollen, wax and nectar within colonies, pollen and nectar residues from plants, in pollen loads on bees returning to the hives and in adult workers (Thompson 2012). Pesticide regulations so far focused mainly on protection of bees against direct poisoning (Thompson and Wilkins 2003, Desneux et al., 2007). The direct poisoning is now regulated and prevented by the implementation of European Council Directive 91/414 in Europe, and the Federal Insecticide, Fungicide and Rodenticide Act in the US (Desneux et al. 2007, vanEngelsdorp and Meixner 2010). The standard approaches for determination of acute pesticide toxicity in bees are the calculation of the LD50 (median lethal dose) or LC50 (median lethal concentration) of a given substance with respect to adult bees or larvae. In spite of more or less controlled protection against direct poisoning, massive dying of honey bees is still present. For this reason many studies are focusing to the chronic sub-lethal exposure of xenobiotics causing a variety of sub-lethal effects on bees (reviewed by Desneux 2007) which are physiological and behavioral, affecting the honeybee colony as a whole, resulting in the perturbations of learning and communication ability. Even more, as many pesticides are applied

together, scientists are arguing for years that toxic exposures to pesticides should be measured as they would normally occur, in combination with one another. The most intriguing or concerning aspect of pesticide mixtures is the opportunity for complex interactions such as a synergy when the administration of one chemical increases the toxicity of another. There are relatively few experimental data regarding synergistic effects of pesticides on honeybees, but in some cases pesticide mixtures, particularly with insecticides, have been shown to be synergistic, with reported increases in toxicity of up to100-fold (Thomson 1996). However, the effects of pesticide exposure on colony health is not systematically monitored, and the Environmental Protection Agency (EPA) does not require data on sub-lethal or synergistic effects for pesticide registration (NAS, 2009) therefore this specific issue warrants special attention.

This review focuses on the mechanisms and molecular targets of xenobiotics in honeybees which could be the basis of their synergism, especially insecticides which are the most potentially dangerous for honeybees. Since of greater potential to cause underestimation of the risk posed to the honeybee colonies decline the modes of synergisms of xenobiotics known so far are emphasized and summarized in this section of our review. The aim is to exemplify the possible complex mechanisms of their interaction.

Mechanisms and effects of xenobiotics

Agrochemical pesticides

The agrochemical pesticides affecting honey bee colonies are fungicides, herbicides and insecticides, applied to crops (Johnson et al. 2010). The large number of commercial pesticides used worldwide, whether based on natural products or being entirely of synthetic origin, act on relatively few, perhaps 95 biochemical targets in pest insects, weeds, and destructive fungi (Casida 2009). Herbicides in general are blocking photosynthesis, carotenoid synthesis, or aromatic and branched chain amino acid synthesis essential in plants. Many fungicides inhibit ergosterol (the fungal sterol) or tubulin biosynthesis or cytochrome c reductase. Others disturb basic cellular functions (Casida 2009).

The pesticides that represent a main threat to the honeybees are **insecticides**. Many of the most effective insecticides in current use act on the insect nervous system (Narahashi 1992, Bloomquist 1996).Others are insect growth regulators (Tasei 2001, Thomson et al. 2005). The growth regulating insecticides are functioning as juvenile hormone analogues (fenoxycarb), chitin synthesis inhibitors (diflubenzuron), ecdysteroid synthesis inhibitors (azadirachtin) and ecdysteroid analogues (tebufenozide) (Tasei 2001, Thomson et al. 2005).The main nerve targets of current insecticides are voltage-gated sodium channels, an enzyme acetylcholinesterase (AChE) and receptors for neurotransmitters: L-glutamate-gated chloride channels working as glutamate receptors (GluRs), ionotropic γ-aminobutyric acid (GABA) receptors(GABARs) binding GABA and nicotinic cholinergic receptors (nAChRs) stimululated by acetylcholine (ACh) (Coats 1990, Fukuto 1990, Zlotkin 1999, Bloomquist 2003, Raymond-Delpech et al. 2005, Wolstenholme and Rogers 2005, Davies et al. 2007, Jeschke and Nauen 2008).Voltage-gated sodium channels are molecular targets for three big groups of insecticides, **pyrethroid, DDT-type** and **organ chlorine** insecticides (Coats 1990). These channels mediate the transient wave of sodium entry spreading along the nerve axons and dendrites, carrying the action potential along these structures and are ubiquitous in the honeybee nervous system (Sattelle and Yamamoto 1988, Narahashi 1992). All three groups of insecticides cause death due to hyperexcitation of the nerves, but in slightly different way. Pyrethroid pesticides by binding to voltage-gated sodium channels induce hyperexcitation that results from prolongation of the open phase of sodium gate function results in neurotoxic effects such as tremors and convulsions. The DDT-type insecticides, DDT (dichloro diphenyl trichloroethane) and DDT analogues (N-alkylamides, dihydropyrazoles), act primarily on the peripheral nervous system (Coats 1990). The mechanism of DDT is the prevention of the deactivation or closing of that gate after activation and membrane depolarization. The result is a persistent leakage of Na+ ions through the nerve membrane, creating a destabilizing negative afterpotential. The hyperexcitability of the nerve is the consequence of trains of repetitive discharges in the neuron after a single stimulus and/or occur

spontaneously (Coats 1990).The acute toxic effects in animals of organ chlorine insecticides are also due to hyperexcitation in the nervous system and death is frequently recognized as respiratory failure after the disruption of nervous system function (Coats 1990).

Organophosphate and **carbamate** insecticides are inhibiting the action of AChE (Fukuto 1990). AChEis an enzyme that terminates the synaptic actions ofACh, the important neurotransmitter of sensory neurons and interneurons of insect brain which is necessary for sensory-input processing and learning in honey bee (Massoulie et al. 1993, Homberg 1994, Weinberger 2006). AChE is widely distributed in the insect brain, the thoracic and abdominal segments and the abdominal ganglia (Kreissl in Bicker 1989, Thany et al. 2010).The potential target sites for organophosphate and carbamate insecticides in the honeybee brain are the optic lobes, antennal afferents projecting into the dorsal lobe, fibers connecting the two brain hemispheres, and within the protocerebrum and the mushroom bodies where AChE is highly expressed (Kreissl in Bicker 1989). AChE was found also in the compound eye and ocelli (Kral 1980, Kral and Schneider 1981).The inhibition of AChE by organophosphate and carbamate insecticides causes irreversible blockage leading to accumulation of the enzyme which results in overstimulation of cholinergic receptors (Fukuto 1990). As ACh is a major neurotransmitter of insect nervous system (Homberg 1994) the inhibition of AChE could cause a systemic failure in the insect body. Widely used organophosphate as hive varroacides is coumaphos.

Insecticides that act selectively on insect nAChRs as potent agonists are **neonicotinoids** (Jeschke and Nauen 2008). Among ionotropic receptors affected by insecticides, nAChRs are the most abundant excitatory postsynaptic receptors (Sattelle 1980).The central nervous system of insects is rich in nAChRs more so than any other organism (Jones and Sattelle 2010).They are located postysinaptically and directly activated by ACh, released from presynaptic cholinergic neurons facilitating fast excitatory synaptic transmission (Thany et al. 2010).In the honeybee brain the highest binding site densities for nAChR are localized in the suboesophageal ganglion, the optic tubercles, optic lobes medulla and lobula, antennal lobes, dorsal lobes and the α-lobes of the mushroom bodies (Scheidler et al. 1990). Neonicotinoids cause excitation of the neurons and because of a high concentration of nACh receptors in honeybees the eventual paralysis could be very profound occurring at low concentration of neonicotinoids, leading to death.

The insecticides that interfere with GABARs are **pyrethroids and phenylpyrazole insecticides** (Raymond-Delpech et al. 2005, Davies et al. 2007). In insects GABARs are associated with neurotransmitter GABA mediating inhibitory synaptic transmission in the nervous system and at nerve-muscle junctions (Homberg 1994). In the central nervous system of honeybees the neurotransmitter GABA is generally present in neuropil, especially in structures that are associated with learning and memory, such as antennal lobe and the mushroom body and the optic lobe (Schafer and Bicker 1986, El Hassani et al. 2009). The presence of the neurotransmitter GABA in the honeybee brain was shown mainly for local interneurons and less in the projection neurons. In the brain and subesophageal ganglion only minority of neurons contained GABA (Bicker et al. 1985, Meyer et al. 1986, Schafer and Bicker 1986). By targeting the GABARs which are chloride channels pyrethroids and phenylpyrazole insecticides disrupt normal neuronal influx (e.g., passage of chloride ions) and, at sufficient doses, causing excessive neural excitation, severe paralysis, and death (Cole et al. 1993, Gunasekara et al. 2007). Most known representative of phenylpyrazole insecticides is fipronil and widely used pyrethroid as hive varroacides is tau-fluvalinate. Pyrethroids are very complex group regarding the molecular mechanisms of their functioning, because they don't bind only to GABA-gated chloride channel, but they can also interfere with other molecules such as calcium regulation. They could inhibit both Ca-ATPase and Ca-Mg ATPase (Coats 1990). In this respect direct effects on neurotransmitter release have been observed, as well as the inhibition of Ca2+ uptake. However, they have also various secondary targets such as signal transduction pathways by altering the protein phosphorylation cascade that may result, among other things, in programmed cell death (Ray and Fry 2006). In mammals a variety of different effects of pyrethroids were discovered like modulation of protein

phosphorylation, voltage-gated sodium channels, voltage-gated chloride channels, noradrenaline release, membrane depolarization, GABA-gated chloride channels, nicotinic receptors, mitochondrial complex I, apopotosis induction, voltage-gated calcium channels, lymphocyte proliferation, volume-sensitive anion channels, calcium ATP-ase, intercellular gap junctions and chromosomal damage, but many of these effects were not shown for insects (Ray and Fry 2006).

The insecticides that activate GluRs which bind neurotransmitter L-glutamate are **avermectin** and **milbemycin** (Raymond-Delpech et al. 2005, Wolstenholme and Rogers 2005). The distribution of GluRs in the nervous system of honeybees is not known but they probably modulate excitability in the nervous system and muscle cells as neurotransmitter L-glutamate is enriched in these tissues (Cully et al. 1996). Studies performed by Maleszka et al. (2000) and Locatelli et al. (2005) suggested that glutamatergic neurons in the honeybee brain, in particular those found in the mushroom bodies, may be part of the circuitry involved in processing of olfactory memory. In the honeybee, a high level of a glutamate transporter is present in the optic lobes and in restricted areas of the mushroom bodies corresponding to the Kenyon cells of the calyces (Kucharski et al. 2000). GluRs are permeable to chloride ions and the activation of these receptors with insecticides avermectin and milbemycin causes a very long-lasting hyperpolarization or depolarization of the neuron or muscle cell and therefore blocking further function leading to paralysis and death (Wolstenholme and Rogers 2005).

Insecticides have various neural effects in honeybees that were in details reviewed by Belzunces et al. (2012). They impair cognitive functions, including learning and memory, habituation, olfaction and gustation, navigation and orientation. They affect also behavior, including foraging and physiological functions, including thermoregulation and muscle activity.

Acaricides

Commonly used in hive varroacides are amitraz, coumaphosand tau-fluvalinate (Johnson et al. 2010). Amitraz is a formamidine pesticide. The mode of action of formamidine pesticides such in insects is believed to be the toxic effects on a G protein-coupled receptor for a neuromodulator octopamine, working as octopaminergic agonists (Evans and Gee 1980, Dudai et al. 1987). High levels of octopamine in the honey bee brain are associated with increased foraging behavior (Schulz and Robinson 2001). Forager honey bees treated with octopamine increased the reported resource value when communicating via the dance language (Barron et al. 2007). However, the effects of amitraz on foraging activity of honeybees were not investigated, but the acute toxicity of this compound was shown in larvae where it increases apoptotic cell death in the midgut (Gregorc and Bowen 2000). Another popular in hive varroacide is tau-fluvalinate which was initially very effective at controlling Varroa mites by blocking voltage-gated sodium channels (Davies et al. 2007). Tau-fluvalinate was quite promising since it is tolerated by bees in high concentrations due to rapid detoxification by cytochrome P450 monooxygenases, but many Varroa populations are now resistant (Lodesaniet et al. 1995, Johnson et al. 2009). However, tau-fluvalinate is not completely harmless, high doses could affect queens to grow smaller and drones to dieuntil reaching sexual maturity (Rindereret et al. 1999, Haarmann et al. 2002). As the efficacy of tau-fluvalinate against Varroa was beginning to decrease, coumaphos, an organophosphate pesticide, was starting to be used (Elzen and Westervelt 2002). Although honey bees can tolerate similar to tau-fluvalinate therapeutic doses of coumaphos, probably as a result of detoxicative P450 activity (Johnsonet et al. 2009), negative effects from coumaphos exposure were observed. Queens exposed to coumaphos were smaller, suffered higher mortality and were more likely to be rejected when brought into a colony (Haarmann et al. 2002, Collins et al. 2004, Pettis et al. 2004). Drone sperm viability was lower in stored sperm collected from drones treated with coumaphos (Burley et al. 2008). Coumaphos also affects food transfer between workers of honeybee (Bevk et al. 2011). Fenpyroximate is a pyrazole acaricide that presumably kills mites through the inhibition of electron transport in the mitochondria at complex I, thereby interfering with energy metabolism (Motoba et al.1992). It was found that chronic exposure to fenpyroximate causes the increased generation of reactive oxygenspecies (Sherer et al. 2007).

Two monoterpenoid components of plant-derived essential oils, thymoland menthol, are used for control of Varroa and tracheal mites. They were found to be among the most toxic of all terpenoids tested when applied to honey bees as a fumigant (Ellis and Baxendale 1997).The thymol molecular targets include binding to octopamine receptors (Enan 2001) and AChE (Priestley et al. 2003), but also insect tyramine and GABA receptors (Blenau et al. 2011).Receptor activation leads to changes in the concentration of intracellular second messengers such as cAMP or InsP3/Ca2+. Thymol could affect honeybees inducing brood removal (Marchetti and Barbattini 1984, Floris et al. 2004) and the increase of queen mortality (Whittington et al. 2000). Exposure to thymol was shown to decrease phototactic behavior in the honeybee (Bergougnoux et al. 2013).

Among organic acids, formic acid and oxalicacid are used as varroacides. Formic acid is inhibiting electron transport in the mitochondria binding of cytochromec oxidase in mites and may produce a neuroexcitatory effect on arthropod neurons (Keyhani and Keyhani 1980, Song and Scharf 2008). Formic acid can reduce worker longevity (Underwood and Currie 2003) and harming brood survival (Fries 1991).The mode of action of oxalic acid against Varroa is unknown, but in mammals it interferes with mitochondrial electron transport leading to increased production of reactive oxygen species and to kidney toxicity (Cao et al. 2004, Meimaridou et al. 2005). Repeated treatment of colonies with oxalic acid can result in higher queen mortality and a reduction in the amount of sealed brood (Higes et al. 1999). The midguts of honey bees fed oxalic acid in sugar water exhibited an elevated level of cell death (Gregorc and Smodisskerl 2007).Recent studies are focusing on molecular mechanisms underlying the sub-lethal effects of in-hive acaricides on honey bees. Using a gene expression profiling Boncristiani et al. (2012) found that thymol, coumaphos and formic acid are able to alter detoxification gene expression pathways, components of the immune system responsible for cellular response and developmental genes. This study indicates that these acaricides could significantly influence the health of individual honey bees and entire colonies (Boncristiani et al. 2012).

Mechanisms and factors influencing the synergy of xenobiotics applied to honeybees

Understanding the toxicity and synergy of chemicals in organisms requires considering the molecular mechanisms involved as well as the relationships between exposure concentration and toxic effects with time (Tennekes and Sánchez-Bayo 2013).In addition, the relevance of synergy of xenobiotics is a subject to understanding the routes of application, the way of transportation to target molecules in the tissue and the metabolism of pesticides in the target organism, all having a profound influence on the concentration and chemical structure of active substances at target sites. The analysis of the studies when monitoring the residues in honeybees following in-hive treatments or pesticide applications revealed that the highest exposure routes were sprayed and systemic seed and soil compound treatments to which preferentially foraging bees are exposed during collecting contaminated nectar and the direct exposure to acaricides used in beehives (Thomson 2012). This is probably due to the availability of relatively high concentration of agricultural pesticides and in-hive compounds, but also the time between pesticide application to crops and bee exposure is very important as many pesticides degrade or dilute in the environment. The importance of other routes of exposure such as dusts produced during sowing of treated seeds, water from puddles or guttation droplets and beeswax might be relevant but data about these are limited. The final actions of xenobiotics are greatly dependent on the mode of exposure, acute, sub-chronic and chronic, defining the nature and the intensity of their effects. Metabolism of xenobiotics elicited by intrinsic enzymes is remarkably important as it could result in the elevation or decrease of their toxicity or it could produce different effects. Chemical interactions between xenobiotics in the mixture are also possible, causing the changes in chemical structures of particular substance. There are also other factors such as physiological states of the organisms including age, the season and the capacity of immune system that have impact on synergism (Thomson 2012). For example, the immune system of honeybees could be profoundly affected by various pathogens, bacterial, fungal

and viral pathogens as well as ecto- and endoparasites that in many cases elevate the toxicity of xenobiotics. Most of the studies in honeybees have focused on the synergisms at the level of midgut enzymes when certain xenobiotic inhibit the detoxifying ability of these enzymes and potentiate the toxicity of another substance, but the synergism at target site is poorly investigated.

The synergism at the level of midgut detoxifying enzymes

Probably the most frequent way of the transfer of xenobiotics into honeybee tissue is the consummation of contaminated nectar and absorption in the midgut thought the midgut wall into the hemolymph, but also passage thought cuticle and sometimes inhalation of vaporous compounds is possible. In the midgut of the honeybee xenobiotics are metabolized by enzymes glutathione-S-transferases (GSTs), cytochrome P450 monooxygenases (P450s) and carboxyl/cholinesterases (CCEs) (Scott and Wen 2001, Enayati et al. 2005, Wheelock et al. 2005). These enzymes metabolize pesticides by different mechanisms, but P450s are probably the most important for honeybees as they play a significant role in the detoxification of phytochemicals present in the nectar, honey and pollen that bees consume (Mao et al. 2009). They catalyze a range of reactions including oxidation and demethylation which decrease pesticide activity or produce active metabolites (Scott and Wen 2001).For example, they convert the thion to oxon forms of organophosphorus pesticides or change neonicotinoid thiamethoxam to clothianidin. P450s can also oxidaze aromatic rings of tau-fluvalinate and flumethrin used in varroa control (Ortiz de Motellano and De Voss 2005). GSTs in insects can metabolize insecticides by facilitating their reductive dehydrochlorination or by conjugation reactions with reduced glutathione, to produce water-soluble metabolites that are more readily excreted. In addition, they contribute to the removal of toxic oxygen free radical species produced through the action of pesticides (Enayati et al. 2005).Carboxylesterases (CaEs) are hydrolazes and catalyze the hydrolysis of carboxyl esters of three different classes of agrochemicals, pyrethroids, organophosphates and carbamates via the addition of water (Wheelock et al. 2005). The selective toxicity of xenobiotics is affected by the ratio and the levels of metabolizing enzymes which fluctuate in different insect species and also in individual organism. The level of enzymes could be affected also by the season, the study on winter honeybees demonstrated reduced levels of P450-mediated detoxification since the synergism between pyrethroid deltamethrin and the P450-inhibiting fungicide prochloraz was much reduced during winter periods (Meled et al. 1998).

By far the majority of the studies of pesticide synergism in honeybees have focused to P450 enzymes that are inhibited by specific pesticides mostly by monitoring the toxicity calculation of the LD50 or LC50 (Table 1.).The developers of insecticide synergists have often exploited inhibition of P450s activity to prolong the efficacy of pesticides which are otherwise rapidly detoxified. It was shown that P450-inhibitors elevated toxicity of pyrethroids (cyfluthrin, permethrinand tau-fluvalinate), neonikotinoid insecticides(imidacloprid, acetadimiprid, thiacloprid), and carbamate insecticide carbaryl (Georghiou and Atkins Jr. 1964, Yu et al. 1984, Hagler et al. 1989, Iwasa et al. 2004, Johnson et al. 2006). It was also found that the classic P450 inhibitors PBO synergize with varroacides tau-fluvalinate and coumaphos at high levels but other inhibitors have minor effect (Johnson et al., 2009, Johnson et al., 2013). Many examples of synergy have been reported between EBI (ergosterol biosynthesis inhibitor) fungicides such as prochloraz, propiconazole, epoxiconazole, carbendazimand insecticides due to the fungicide inhibitory action on P450s. This was the case with neonicotinoids (acetamiprid, thiacloprid, imidacloprid) and pyrethroid insecticides (deltamethrin, lambda-cyhalothrin, alphacypermethrin) (Pilling 1992, Meled et al. 1998, Vandame and Belzunces 1998a, Vandame and Belzunces 1998b, Papaefthimiou and Theophilidis 2001, Thompson and Wilkins 2003, Schmuck et al. 2003, Iwasa et al. 2004, Thompson 2013).The effects of EBI fungicides on the contact toxicity of the active ingredients of the pyrethroid varroacides flumethrin and tau-fluvalinate are synergized by the fungicides with relatively high increases in toxicities (Thompson and Wilkins 2003). Another EBI fungicide prochloraz which is also a P450s inhibitor elevated the toxicity of the acaricides coumaphos and fenpyroximate

(Johnson et al. 2013). The studies on synergism between insecticides in honeybees were rarely conducted, most of them between in-hive acaricides. Johnson et al. (2009) observed a large increase in the toxicity of tau-fluvalinate to bees that had been treated previously with coumaphos, and a moderate increase in the toxicity of coumpahos in bees treated previously with tau-fluvalinate. These compounds were chosen due to their low toxicity to honey bees which were attributed to rapid detoxification mediated by P450s. The synergisms occurred also between in-hive miticides coumaphos, thymol, amitraz, fenpyroximate and oxalic acid (Johnson et al. 2013). The observed synergism was explained as a result of competition between miticides for access to detoxicative P450s (Johnson et al. 2009).See the Table 1. for the list of synergisms of xenobiotics observed in honeybee.

The synergisms were found also for carbamate insecticides (carbaryl, carbofuran) and herbicide atrazine but the mechanism of this synergy is unknown (Sonnet et al. 1978). The synergy between monoterpenoid thymol and tau-fluvalinate or coumaphos was observed and was explained to be the consequence of the P450s inhibitory activity of thymol, but thymol inhibitory property was shown only in human liver microsomes but not for honeybee midgut (Johnson et al. 2013).

In other organisms, the synergisms were studied between insecticides and insecticide/herbicide at the level of detoxifying enzymes. The interactions such as a competition with metabolic enzymes esterases are possible that are maybe not very significant for honeybees since it was shown that the role of these enzymes participating in the detoxification of xenobiotics is minor. It was also shown that certain organophosphate insecticides could bind to the active site associated with esterase enzymes responsible for detoxification of pyrethroid-based insecticides and so organophosphate insecticides may be considered useful synergists for pyrethroids (Cloyd 2011).The synergisms at the level of detoxifying enzymes was described also for organophosphates and pyretroids, P450 activated by organophosphates decrease the organism's ability to detoxify pyretroids due to esterases inhibition, so greater than additive toxicity is often observed (Hernández et al. 2013). Recent studies have demonstrated the potentiating effects of triazine herbicides, such as atrazine to the toxicity of organophosphates when these herbicides stimulate P450 activity by increasing the rate of bioactivation of organophosphates resulting in the potentiation of the cholinesterase inhibiting property of organophosphates (Hernández et al. 2013).

It seems that the regulation of the P450s in honeybees is unique. Contrary to other insects, in the honey bee these enzymes are rarely induced by a substrate itself. The honeybee genome has substantially fewer protein coding genes for xenobiotic detoxifying enzymes than *Drosophila melanogaster* and *Anopheles gambiae* (Claudianos et al. 2006) and many researchers failed to demonstrate an increase of midgut detoxifying enzymes induced by xenobiotics (Yu et al. 1984). Even exposure to phenobarbital which is an inducer of P450s showed no alterations in the expression of many of P450 genes tested in honey bees (Mao et al. 2011). Only two studies indicated the increase in P450 activity in honeybees. Application of tau-fluvalinate and coumaphos elevated specific detoxifying P450 enzymes CYP9Q1, CYP9Q2, and CYP9Q3 and benzo(α)pyrene monooxidase activity in honey bee guts was induced by exposure to benzo(α)pyrene itself and by the in-hive acaricides tau-fluvalinate and cymiazole hydrochloride (Kezic et al. 1992, Mao et al. 2011). As it has been at first suggested that reduced diversity of detoxification enzymes may contribute to the sensitivity of honey bees to certain pesticides (Claudianos et al. 2006) the importance of midgut detoxifying enzymes P450 in honeybees was highlighted by the studies with specific P450-inhibitors. Two studies indicate that GSTs and CaEs are active detoxifying enzymes in honeybees but they play a relatively minor role in detoxification as compared to P450s (Johnson et al. 2009, Iwasa et al. 2004). The CaEs inhibitor DEF (S,S,S-tributylphosphorotrithioate) and GSTs inhibitor DEM (diethyl maleate) were shown to increase the toxicity of certain pyrethroids and neonicotinoids but this effect was significantly smaller that for the P450 inhibitor PBO (piperonyl butoxide) (Iwasa et al. 2004). Recently the interesting study performed by Johnson et al. (2012) suggested that regulation of honey bee P450s is affected by chemicals occurring naturally in the hive environment in the nectar, pollen and propolis since only quercetin, a common pollen

Table 1: The list of synergisms of xenobiotics in honeybee *Apis mellifera* and proposed mechanisms.
Tabela 1: Seznam sinergizmov med ksenobiotiki v medonosni čebeli *Apis mellifera* in predlagani mehanizmi.

Xenobiotic	Xenobiotic (P450 inhibitor)	Reference
Mechanism of synergy: inhibition of P450 detoxifying enzymes		
pyrethroid insecticides	***classical P450-inhibitor***	
cyfluthrin	piperonyl butoxide	(Johnson et al. 2006)
permethrin	piperonyl butoxide	(Hagler et al. 1989)
lambda-cyhalothrin	piperonyl butoxide	(Johnson et al. 2006)
tau-fluvalinate	piperonyl butoxide	(Johnson et al. 2006; Johnson et al. 2013)
neonicotinoid insecticides	***classical P450-inhibitor***	
imidacloprid	piperonyl butoxide	(Iwasa et al., 2004, Johnson et al. 2012)
acetadimiprid	piperonyl butoxide	(Iwasa et al. 2004)
thiacloprid	piperonyl butoxide	(Iwasa et al. 2004)
carbamate insecticide	***classical P450-inhibitor***	
carbaryl	piperonyl butoxide	(Georghiou and Atkins Jr. 1964)
hive varroacides	***classical P450-inhibitor***	
tau-fluvalinate	piperonyl butoxide	(Johnson et al. 2009, Johnson et al. 2013)
coumaphos	piperonyl butoxide	(Johnson et al. 2009, Johnson et al. 2013)
fenpyroximate	piperonyl butoxide	(Johnson et al., 2013)
neonicotinoid insecticides	***EBI (ergosterol biosynthesis inhibitor) fungicides***	
acetamiprid	epoxiconazole, propiconazole, triadimefon, triflumizole, uniconazole-P	(Iwasa et al. 2004)
thiacloprid	prochloraz, propiconazole, tebuconazole, triflumizole	(Schmuck et al. 2003, Iwasa et al. 2004)
imidacloprid	propiconazole, triflumizole	(Iwasa et al. 2004)
pyrethroid insecticides	***EBI (ergosterol biosynthesis inhibitor) fungicides***	
deltamethrin	difenoconazole+carbendazim, prochloraz, prochloraz+ difenoconazole 850	(Belzunces and Colin 1993, Colin and Belzunces 1992, Papaefthimiou and Theophilidis 2001, Vandame and Belzunces1998b, Vandame and Belzunces 1998a)
lambda-cyhalothrin	difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole, thiophanate-methyl	(Thompson and Wilkins 2003)
alphacypermethrin	difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole	(Thompson and Wilkins 2003)
hive varroacides	***EBI (ergosterol biosynthesis inhibitor) fungicides***	
coumaphos	prochloraz	(Johnson et al. 2013)
flumethrin	carbendazim, difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole, thiophanate-methyl	(Thompson and Wilkins 2003)

Xenobiotic	Xenobiotic (P450 inhibitor)	Reference
tau-fluvalinate	carbendazim, difenconazole, flusilazole, prochloraz, propiconazole, tebuconazole, thiophanate-methyl, myclobutanil, metconazole, fenbuconazole,	(Thompson and Wilkins 2003, Johnson et al. 2013)
fenpyroximate	prochloraz	(Johnson et al. 2013)
hive varroacides	***hive varroacides***	
coumaphos	tau-fluvalinate	(Johnson et al. 2009, 2013)
thymol	tau-fluvalinate, coumaphos	(Johnson et al. 2013)
amitraz	tau-fluvalinate, coumaphos, fenpyroximate	(Johnson et al. 2013)
fenpyroximate	tau-fluvalinate, coumaphos	(Johnson et al. 2013)
Mechanism of synergy: increased oxidative stress		
hive varroacides	***Fungicides (mitochondrial inhibitors)***	
tau-fluvalinate	pyraclostrobin, boscalid	(Johnson et al. 2013)
fenpyroximate	pyraclostrobin	(Johnson et al. 2013)
Unknown mechanism of synergy		
oxalic acid	tau-fluvalinate, fenpyroximate, amitraz, thymol	(Johnson et al. 2013)
herbicide atrazine	carbamate insecticides (carbaryl, carbofuran)	(Sonnet et al. 1978)
thio and dithiophosphoric ester pesticides – ethyl parathion, dimethoate, dialifos	coumaphos varroacide	(Lienau 1990)
thiacloprid (neonicotionoid)	fungicides cyprodinil, tolyfluanid	(Schmuck et al. 2003)
alphacypermethrin, lambda-cyhalothrin	fungicide chlorothalonil fungicide chlorothalonil	(Thompson and Wilkins 2003)

and honey constituent, reduced tau-fluvalinate toxicity. Bees fed with extracts of honey, pollen and propolis showed elevated expression of three CYP6AS P450 genes. Non-naturally occurring inducers of cytochrome P450 enzymes did not alter the toxicity of certain xenobiotics and it seems that a wide range of synthetic pesticides do not induce in bees. It is now clear that certain substances found in bee products such as quercetin, p-coumaric acid, pinocembrin, and pinobanksin 5-methyl ether naturally elevate the levels of bee detoxifying enzymes P450 and probably helping bees to resist the toxicity of certain xenobiotics (Johnson et al. 2012, Mao et al. 2013).

The synergism of xenobiotics working at the same targets

Although the basic molecular mechanisms of most xenobiotics are more or less known, the possible mechanisms of their synergy at primary target sites in honeybees are unexplored. One of plausible mechanism of this synergy is that effects at the site of toxic action include increased response of the site (such as a receptor) following initial pesticide exposure and according to this direct synergistic effect could be predicted for substances that have similar targets (Thomson 1996). In this respect only one study in honeybees was performed, on semi-isolated heart (Papaefthimiou and Theophilidis 2001).In this study the synergistic effect was observed between EBI fungicide

prochloraz and pyrethroid insecticide deltamethrin which rapidly decreased the frequency and the force of the cardiac contractions with marked effects at 0.01 μM, equivalent to internal doses of 4–5 μg kg^{-1} body weight. Prochloraz showed to be more cardiotoxic than deltamethrin, what seemed surprising since deltamethrin is a neurotoxic substance whereas prochloraz is an inhibitor and an inducer of detoxifying enzymes P450. So, authors concluded that there must be the neural basis of the deltamethrin prochloraz synergy. Belzunces et al. (2012) suggested that the basis of their synergy is the interaction of these two pesticides with shared molecular targets, such as ATPases, potassium and calcium channels.

The existence of the synergy of insecticides at primary target sites was demonstrated also in the cockroach *P. americana*, in the cercal-afferent giant-interneuron synapses of the terminal abdominal ganglion (Corbel et al. 2006). Authors demonstrated that pyrethroid permethrin and carbamate propoxur insecticides applied together increased drastically the ACh concentration within the synaptic cleft, which thereby stimulated a negative feedback of ACh release mediated by presynaptic muscarinic receptors causing the synergism. Johnson et al. (2013) demonstrated 5-fold increase in the toxicity of tau-fluvalinate by amitraz pretreatment in honeybees. Interactions between formamidines and pyrethroids are known in other insects and may be due to synergism at the target site through cooperative binding (Liu and Plapp 1992).It was shown that formamidine pesticides working as octopaminergic agonists change the binding properties of pyrethroid insecticides to nerve membrane sodium channels. This mechanism could be also the cause for the synergism of tau-fluvalinate and amitraz in honeybees. Thomson (1996) reported another possible mechanism of pesticide interaction, when esterases can act as irreversible binding sites for organophosphate and carbamate insecticides, reducing the levels available to bind to AChE within the brain. Thus, prior exposure to an organophosphate may result in a reduction in the number of available binding sites and an increase in the blood levels of free pesticide. All these mechanisms mentioned above are possible for honeybees, but they are not explored. The lack of similar studies suggests that the mechanisms of synergisms of xenobiotics at primary target sites in honeybees are very much ignored and underestimated and a need for additional studies is unavoidable.

Conclusions

Bees are very often exposed to mixtures of products applied to plants on which they forage such as fungicides, herbicides and insecticides and in addition very high levels of varroacides may be present within colonies. The very potential risk from most mixtures of these substances is the development of synergisms that can profoundly affect honeybee colonies and may significantly contribute to honeybee colony loss observed in the last 15 years. This risk is underestimated and relatively few relevant studies were performed concerning the effects and mechanisms of synergy of different xenobiotic combinations. The understanding the mechanisms of synergy between xenobiotics is very important for the restriction of the use of defined mixtures and also for the prediction of potential toxicity of newly developed substances in agriculture and apiculture. Many observable effects are induced by xenobiotics such as alternation of cognitive functions, behavior or integrity of physiological functions, many of them unambiguously explained by the mechanisms of xenobiotic actions at primary target sites. In spite of these physiological mechanisms of action of individual xenobiotics are more or less identified, especially for insecticides, the majority of synergistic effects observed in honeybees is ascribed to the inhibition of detoxifying midgut enzymes P450 involved in xenobiotic metabolism. Even more, as most of the studies focused on synergistic effect of mixure xenobiotic/P450 inhibitor, only few were performed on insecticide/insecticide interactions. Johnson et al. (2013) proposed that the synergistic interactions occur when the compounds work through different modes of action, but few experiments in insects studying the synergism of insecticides at target sites suggest that the synergism is also possible for substances working through the same mode of action, at least when they are working on the same system such as cholinergic synapse. Therefore, the aspect of mechanisms of synergism at the similar targets is underestimated since only one study was performed in honeybee and

therefore this issue demands extra investigation. The improved knowledge of the mechanisms of pesticide and bee-hive compound interactions would prevent the negative impact on beneficial organisms like honeybees.

Povzetek

Medonosne čebele so med iskanjem hrane pogosto izpostavljene različnim ksenobiotikom, večinoma so to fitofarmacevtska sredstva in panjske kemikalije. Čebele v zadnjih 15 letih množično umirajo, vzrok za to naj bi bila tudi uporaba ksenobiotikov. Številna fitofarmacevtska sredstva se uporablja hkrati in znano je, da lahko pride do sinergističnih interakcij v organizmih. Tveganje za nastanek sinergizmov je podcenjeno in narejenih je relativno malo študij na čebelah o učinkih in mehanizmih sinergizmov različnih kombinacij ksenobiotikov. Razumevanje mehanizmov sinergizmov ksenobiotikov je zelo pomembno za nadzor nad uporabo definiranih mešanic in napovedovanje potencialne toksičnosti novih ksenobiotikov v kmetijstvu in čebelarstvu. Pregledni članek se osredotoča na učinke, mehanizme in molekulske tarče ksenobiotikov v medonosnih čebelah in osvetljuje primere mehanizmov nastanka sinergizmov. Opisani so tudi drugi dejavniki,ki vplivajo na njihov nastanek, okoljski in fiziološki, poudarek je na detoksifikacijskih encimih medonosne čebele. Najbolj nevarni za čebele so insekticidi, ki delujejo predvsem na nekaj različnih živčnih molekularnih tarč in tako motijo delovanje živčnega sistema, kar vpliva na vedenje, kognitivne funkcije in fiziologijo čebel. Glavne živčne tarče insekticidov so napetostno odvisni natrijevi kanalčki, encim acetilholinesteraza, glutamatni receptorji, receptorji za gama-aminomasleno kislino in nikotinski receptorji. Znane skupine insekticidov so piretroidi, DDT, DDT podobni insekticidi, organofosfati, karbamati, fenilpirazolni pesticidi ter neonikotinoidi. Kljub temu, da so tarče delovanja posameznih ksenobiotikov,še posebej insekticidov, precej znani, raziskovalci večino sinergijskih učinkov v čebelah razlagajo z inhibicijo črevesnih detoksifikacijskih encimov P450, ki presnavljajo ksenobiotike. Večina študij je bila namreč narejena z mešanicami ksenobiotik/zaviralec encimov P450. Glavni zaviralci encimov P450 so specifični inhibitorji, ki jih dodajajo fitofarmacevtskim sredstvom za podaljšanje učinka ter nekateri fungicidi. Študij na čebelah, s katerimi bi raziskovali sinergizem med insekticidi, skoraj ni. Čeprav so sinergizmi ksenobiotikov, še posebej insekticidov, na primarnih ciljnih tarčah pri čebelah možni, saj so bili prikazani pri drugih organizmih, je ta vidik podcenjen. Narejena je bila samo ena raziskava mehanizmov na tarčnem mestu pri medonosni čebeli, pa še to med insekticidom in fungicidom.

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