Chapter 5

Biomonitoring the Environmental Quality by Bees

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/61616

Abstract

Modern farming techniques have increased the crop yield, but natural habitats of the pollinator were destroyed, affecting their populations compared to native vegetation. A simple, low-cost, and efficient way to determine the presence of insecticide residues from farming is the honeybee as a bioindicator. However in Brazil, there is another species of bee, the stingless bees. The insecticide toxicity analyses the beneficial insect species as pollinators which are performed to the *Apis mellifera*. Stingless bees are native to tropical and subtropical zones, and they are more sensitive to pesticides than honeybees. We present some results of contamination in these bees compared to Africanized honeybees, and pose an important question: Why does the pesticide industry not make assays with stingless bees too? When insecticides were in larger concentrations, bees did not feed. When the concentration of the insecticide was smaller, Africanized honeybees consumed the polluted honey, resulting in the death of some. Finally, we report several experiments concerning honeybees, and mainly stingless bees, and the effect of pesticides in them; results show stingless bees are more sensitive than honeybees. Our Bee Research Group studied this point, and we hope to contribute for understanding this relation between bee, pesticide, and environment.

Keywords: Pesticides, fipronil, thiamethoxam, bee, bioindicators

1. Introduction

Stingless bees are among the most common pollinators in tropical environments. Plant reproduction and pollinators are strongly linked, and the role of bees as pollinators becomes crucial in almost every terrestrial ecosystem. Improper use of pesticides has caused adverse effects on nontarget organisms, such as humans, domestic animals, wild organisms, pollina-

tors, and natural enemies. Insecticides, when used correctly, can control target organisms without compromising the populations of natural enemies and pollinators. Thus, biomarkers have been extensively used to reveal the exposure of organisms to various chemical compounds in the environment.

Decline of pollinator populations is mainly due to the non-sustainable use of chemicals for agricultural production. Excessive insecticide use on crops endangers the populations of pollinating bee species, including stingless bees.

Pollination is a fundamental factor of production in management of crops around the world, and the lack of pollinators for the plants may limit the quantity and quality of the fruit [1]. Therefore, it is essential for good agriculture development with global reach; it also prevents the environmental degradation at local level as well as global level to avoid declining of pollinators [2].

The total number of pollinators is estimated at 40,000 species, with 25,000 being bee species. About 73% of cultivated crops globally are pollinated by some type of bee [3]; 87 of 115 principal global crops benefit significantly from pollinators [4]. A decline of these species or inadequate pollination in some crops may decrease the production by 50%.

The decline of pollinator honeybee *Apis mellifera* worldwide is cause for concern, and several papers have evaluated the harmful effects of insecticides to this bee species. These studies are not frequent in the case of stingless bees because they do not occur in temperate zones [5, 6], although in Brazil we can find a great diversity of these stingless bees. Little research has been done to evaluate insecticide effects on Meliponini, Bombini, and Euglossini bees [7]. The pollinator activity is essential to preserve environment and high yield in agriculture [8].

Stingless bees are among the most common pollinators in tropical environments, and in some regions, these bees are dominant and visit several crops [9]. These insects are a diverse group, which include more than 400 species that present great variability in physiology, morphology, and size, from 0.2 mm in genus *Trigonisca* to more than 20 mm in some species of *Melipona* [10, 11]. The bees belonging to the genus *Tetragonisca* have featured ecological and economic importance. The meliponiculture is a way to preserve the fauna and flora, so the beekeepers can harvest honey, cerumen, and resins. This practice is widely spread, mainly, in the North and Northeast region of Brazil [12], but it increases yearly in other regions of the country.

Bees live in close contact with nature, harvesting pollen, nectar, water, and resins for their colony, so they require that all sources from these resources be pure and without contaminants [13]. The bees are susceptible to several insecticides commonly applied to protect crops, and these insects can be used to biomonitor the environmental quality to detect residues of some insecticides in plants, as well as to detect the toxicity level in bees [14]. Thus, the presence of bees and the quality of their products can be used in environmental biomonitoring, contributing to a better-quality environment for local human populations.

Studying pesticide effects in bees is fundamental; the farmer must learn to select and apply pesticides to control diseases and plagues without risks to the survival of beneficial insects [15]. The presence of pesticide residues is generally detected by physical, chemical, and biological

methods. Theoretically, any organism that is susceptible to an insecticide can be used in these assays, in any environmental sample, and thereby it is possible to make use of biomonitoring to detect some pollutants.

The evaluation and contribution of possible sublethal effects of pesticides in bees have been discussed by scientists and regulation committees [16]. Effects reported by these authors include alterations in learning behavior and the ability of orientation. The alteration in isozyme expression such as esterase that plays a role in metabolism of xenobiotic of bees is another way to use these insects in environmental monitoring.

Due to the importance of bees as pollinators, the usual interaction of beekeeping-agriculture, and the occurrence of areas with natural forests near agricultural areas, it is possible to study the sublethal effects of pesticides in bees. One way to do this is by electrophoretic analysis of esterase; verifying the presence or absence of an alteration in these isozymes can be useful in detecting the environmental contamination in regions in which the bees are visiting or by drifting during the pesticide application.

This review discusses environmental quality using bees to detect residues of pesticides and the alteration that can occur after contamination by sublethal doses of pesticides generally used in agriculture by developed methods with honeybee *Apis mellifera* and stingless bees.

2. Pesticides and biomonitoring

2.1. Pesticides

The first records that man used insecticides to reduce losses by insect attack to the crops are from 1000 B.C. and chemical control of plagues began in twentieth century with chemical 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane (DDT) [17]. Pesticides are classified according to target organisms; fungicides, herbicides, and insecticides are commonly used and are the principal focus of study [18]. The main chemical groups of insecticides of neurotoxic action, general name, and their action site are presented in Table 1.

Insecticides are classified according to their toxicity based on values of lethal dose LD_{50} for any of the routes of contamination (ingestion or contact) presenting the following toxicological categories: **I –** extremely toxic (LD_{50} < 50 mg/kg body weight), **II –** very toxic (LD_{50} 50–500 mg/kg body weight), **III –** moderately toxic (LD_{50} 500–5,000 mg/kg body weight), and **IV –** low toxicity (LD_{50} > 5,000 mg/kg body weight) [19].

Due to the feeding habits of bees, combined with the difficulty of evaluating the effect of chemicals on these insects in the embryonic and larval stages, many researchers seek to associate food with the supply of pesticides. In this sense, the experiment conducted directly provided *A. mellifera* colonies with sugar syrup contaminated with cypermethrin insecticide for 5 consecutive months [22]. During the 18 weeks of treatment, there was a mortality of bees in hives, but some also presented sublethal effects in laboratory tests on samples of bees, such as glycemia, changes in the ATPase enzyme activity, and other physiological disorders and behavioral changes.

Chemical group	General name	Action site
Carbamate	Propamocarb hydrochloride, iprovalicarb, oxamyl	Acetylcholinesterase inhibitors
Organophosphates	Acephate, cadusafos, chlorpyrifos, diazinon, dimethoate, disulfoton, ethione, ethoprophos, fenamiphos, fenitrothion	Acetylcholinesterase inhibitors
Pyrethroids	Acrinathrin, allethrin, alpha-cypermethrin, beta-cyfluthrin, beta-cypermethrin, bifenthrin, cyfluthrin, cypermethrin, d-allethrin, deltamethrin	Modulators of the sodium channel
Neonicotinoids	Acetamiprid, clothianidin, dinotefuram, imidacloprid, Thiacloprid, thiamethoxam	Agonists of nicotinic acetylcholine receptors

Source: Ref. [20].

The registration of all pesticides is performed after toxicity tests. Most of them possess recommendations on toxicity to *A. mellifera* bees. In some localities, there are laws prohibiting the use of pesticides on crops that are blooming (e.g., Ontario, Canada, where it was intended primarily to protect pollinating honeybees in orchards) [21].

Table 1. Main chemical groups of insecticides, general name, and action site

Testing the effect of endosulfan, deltamethrin, baytroid, and sevin, Abramson et al. [23] concluded that none of these products presented repellency effect when provided via food. Except for deltamethrin, the rest were highly toxic to the bees, with mortality 1 hour after feeding. Thompson [24] reported that deltamethrin did not cause mortality of bees but caused sublethal effects at low concentrations, such as hypothermia and loss of memory, making it impossible to return to the colony.

Knowledge about the mechanism of action of insecticides on pollinators is important particularly for bees to use them in a way that minimize lethal and sublethal effects on pollinators [25]. Besides the direct danger of pesticides applied to crops, there is the problem associated with pesticide residues in products of the colony. Thus, highly toxic residues can be found in the hives, jeopardizing the quality of bee products [21].

The food collection of these bees is essential for the maintenance of colony life; however, it exposes the workers to contamination in areas where pesticides have been applied. Therefore, bees require that all sources of their rewards are pure and free from contaminants, including pesticides [26].

The use of conventional pesticides to control pests results in reducing large-scale natural enemies, environmental pollution, resistance to chemical insecticides, as well as increased population of resistant pests [27]. Furthermore, they reduce the diversity of natural enemies of agricultural pests [27], as well as cause a decrease of beneficial insects.

Most synthetic insecticides are toxic to all animals, including man. Although many insecticides can be used safely, few are persistent in the environment and a small number of compounds have mutagenic action, carcinogenic and teratogenic effects in humans and domestic animals [28].

Organophosphate and carbamate pesticides have toxic effects resulting from accumulation of acetylcholine in the synaptic cleft, because the insecticide molecules bind irreversibly to the

catalytic site of acetylcholinesterase, leading to overstimulation of the acetylcholine receptors to produce neurotoxic symptoms [29]. In most bees, acetylcholinesterase is located in the head, especially in compound eyes and ocelli [30, 31]. The detection of anticholinesterase insecticides in bees is difficult to achieve due to their rapid hydrolysis in the body. Thus, one way to detect contamination with these insecticides can be accomplished by inhibition of acetylcholinesterase activity [29]. According to its toxic effect, the organophosphorus compounds can be subdivided into three groups: (a) those highly toxic to bees, such as methyl parathion, malathion, and azodrin, which should not be used in plantations with blossoms; (b) those highly toxic, but with little residual activity, such as mevinphos, that could be applied when bees were not collecting from flowers; and (c) those relatively nontoxic to bees, such as ethion and trichlorfon [32]. Integrated pest management programs determine which insecticides are compatible with a biological control agent and to identify the possible effect on them [32]. Biopesticide studies are frequent in an attempt to reduce the ecological impact that chemicals are causing on the environment [33].

The pesticide group known as bioinsecticides is formed by compounds from plant, animal, fungi, and bacteria, which have allelopathic action in various organisms. *Insect growth regulator insecticides* are biopesticides that have targeted specific characteristics or are stage specific, with a good safety margin for most of the nontarget biota, including invertebrates, fish, and birds, among others. They are relatively safe for humans and domestic animals. Insect growth regulator insecticides mimic juvenile hormone and/or ecdysone in cuticle formation process, and inhibit chitin synthesis in insects and action of the endocrine system [34]. These bioinsecticides are part of a generation of alternative compounds that have been used in agriculture, with a different mode of action of conventional insecticides, acting in specific systems of insects, characterizing them as selective products and low toxicity to mammals [35].

Neonicotinoid insecticides, developed in the 1990s, are registered for use in a wide variety of cultures and effective against insects such as beetles and other insects [36]. The current decline of pollinators has been attributed, at least in part, to the use of neonicotinoids on crops that offer attractive flowers for bees [37].

The class of neonicotinoids originated from the nicotine molecule, extracted from tobacco plants (*Nicotiana tabacum*). The first compound of this class being marketed, imidacloprid, was introduced in Europe and in Japan in 1990 by Bayer CropScience®, which, together with nitenpyram and acetamiprid, represents the cloronicotinil subclass, also known as first-generation neonicotinoids [38].

Commercially, products formulated based on thiamethoxam, the first insecticide of second-generation neonicotinoids, were provided by Syngenta® from 1998, being registered for use on cotton crops, coffee, citrus, soy, and rosettes, are indicated for the control of insect pests of occurrence in the shoot, as aphids, whiteflies, tripods, beetles, and some species of Lepidoptera [39]. Another interesting feature of the molecule is the versatility of use employed by foliar spraying, soil irrigation, and/or seed treatment [40, 41]. They are predominantly used in the application of the seeds in crops such as cotton, canola, and sunflower [42].

Thiamethoxam (3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1, 3,5-oxadiazinan-4-ylidene (nitro) amin) is a crystalline, odorless compound with 139.1°C melting point, low molecular weight (291.72 g.mol^{-1}), relatively high solubility in water (4.1 g.L^{-1} at 25°C), and formula $C_8H_{10}ClN5O_3S$ [40]. The insecticide thiamethoxam is neurotoxic, damages the central nervous system, and inhibits the action of nicotinic acetylcholine receptors of insects by acting specifically on the α subunit of the receptor [43]. These receptors are found in many regions of the brain of bees, including areas involved in learning and memory processes [44]. Unlike acetylcholine, which is hydrolyzed by acetylcholinesterase, these compounds are not degraded immediately; therefore, nervous impulses are transmitted continuously and lead to hyperexcitation of the insect nervous system [45].

Maienfisch et al. [40] reported agonistic activity on nicotinic acetylcholine receptor mimics their docking sites, causing sublethal effects such as loss of memory and orientation [46]. Pettis et al. [47] added that these long-term effects may cause their death or make the colony more susceptible to disease.

In Brazil, a foliar neonicotinoid insecticide is permitted and suitable for various crops, including cotton, citrus, tomato, eucalyptus, apple, strawberry, and soybean [20]. However in 2012, after a series of protests in Europe in order to ban all forms of application of neonicotinoids such as imidacloprid, thiamethoxam, and clothianidin in crops that offer flowers for the bees, the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) declared temporary restriction of the use of the same insecticides and initiated a process of reassessment of these chemicals.

In 2013, the European Food Safety Authority (EFSA) published a document containing a compilation of studies that showed residual values of neonicotinoids found in materials stored in colonies of bees and pollen and nectar crops that offer attractive flowers to them. Inferences were made about the potential risk that the waste could pose to bees. Based on the results, it was concluded that many gaps on the risks that neonicotinoids pose to bees and other pollinators are yet to be understood. Thus, the final position of the European Union was to ban the use of neonicotinoid insecticides for 2 years (2013–2015), until more consistent results are presented [48].

Colony collapse disorder has been one of the biggest concerns about the decline of bee populations in the world [49], and the use of neonicotinoid insecticides has been one of the greatest attributes to this event [46]. The application of this new chemical class of insecticides on crops that offer attractive flowers to bees is growing worldwide [18], and the possibility of its neurotoxic [40] amounts to nontarget organisms, such as bees, justifies its intensification of studies that seek answers to these gaps in knowledge [36].

In recent years, several protests were held in Europe, in general, to ban the use of neonicotinoids on crops that offer flowers for the bees. In Brazil, these substances are sold in commercial formulations, and information use and toxicity can be accessed in the agriculture ministry [20].

2.2. Residues of insecticides

Detoxification is the cellular cleaning process that inactivates toxic compounds that pose risks to cellular health. Organophosphate pesticides, carbamates, and pyrethroids are detoxified by oxidases action with mixed functions, esterases and transferases [50].

The evaluation and contribution of possible sublethal effects of pesticides on bees have been discussed by scientists and regulatory authorities [16]. The effects considered by these authors include changes in learning behavior and orientation ability. Allied to the study of sublethal doses of pesticides, changes in isozyme expression as esterases that would be acting in xenobiotic metabolism of bees is a way of using these insects as bioindicators.

The carboxylesterases and cholinesterase are involved in insecticide resistance, as demonstrated by Whyard et al. [51] and Lee and Lees [52], who observed increased carboxyl esterase activity in *Culex tarsilis* and *Oryzaephilus surinamensis lines*, respectively, resistant to malathion. Among the cholinesterase involved in resistance mechanisms, there is acetylcholinesterase, which is specific to the central nervous system of insects. This esterase regulates acetylcholine levels in the synaptic regions between neurons.

Organophosphate and carbamate pesticides exert acute toxicity by inhibiting acetylcholinesterase (EC 3.1.1.7), a hydrolase serine found in the neuromuscular junction. Acetylcholinesterase is an important enzyme responsible for the rapid hydrolysis of acetylcholine in cholinergic synapses substrate, allowing precise control and modulation of nerve transmission. This leads, in susceptible species, to accumulation of the neurotransmitter acetylcholine and subsequent hyperpolarization of the postsynaptic membrane [45]. Carboxylesterases play a key role in the detoxification of certain hydrolytic organophosphate compounds and play an additional role as alternative binding sites of [53].

2.3. Bee susceptibility to insecticides

Toxicity studies commonly express means of results as LD_{50} and/or LC_{50} (lethal dose and concentration that kills 50% of a population, respectively). Brittain and Potts [54] reported that sublethal effects of pesticide contamination may be more harmful to the colonies in the long run than the lethal effects observed immediately after intoxication. Susceptibility to insecticides may be related to behavioral changes and memory loss that reduce the reproductive success of bees [55].

Bee response to pesticide contamination depends on a number of factors, such as body size, sociality, flight period, floral specialization, and nest behavior [54]. These authors also reported that there was an important correlation between the characteristics presented for each group of bee and environmental conditions provided. For instance, knowledge of the duration of the foraging activity of a bee and the particular plant flowering period will allow the presentation of evidence about the risks of exposure and, consequently, susceptibility for each bee species.

In small concentrations, cells damaged by insecticides can detoxify or be replaced by regenerating cells; however, with high concentrations of insecticides, affected cells are unable to efficiently detoxify and the regenerative cells are also impacted [56]. Another way to detect

bee sensitivity to residues of pesticides is through studies of the change in chromatin by analyzing the critical electrolyte concentration; this technique was developed by Vidal and Mello [57]. The analysis of critical electrolyte concentration checks whether change is occurring in gene expression after contamination with insecticide, leading to changes in the value of the critical electrolyte concentration which is due to inactivation or activation of genes after contamination.

Johnson et al. [58] treated bees with three pyrethroid insecticides (cyfluthrin, lambda-cyhalothrin, and tau-fluvalinate), in which one group was treated with an inhibitor of cytochrome P450 enzyme. These authors found that the toxicity of these insecticides was more significant with the pretreatment, concluding that this enzyme is important in bee tolerance to pyrethroid insecticides.

2.4. Toxicity of insecticides and biomonitoring with bees

Biomarkers have been used extensively to reveal the exposure of organisms to various chemicals in the environment. They are based on physiological, biochemical, anatomical, and behavioral parameters after exposure to pesticides [59].

In the existing literature, there are several studies using *A. mellifera* as a pollutant bioindicator insect in the environment. Toxicity data to verify the susceptibility of *A. mellifera* to 62 insecticides of six classes (carbamates, nicotinoids, organochlorines, organophosphates, pyrethroids, and miscellaneous) were performed [60]. Honeybees can be susceptible to individual insecticides, but they are not highly susceptible to insecticides overall or to specific classes of insecticides. Thereby, there is a great interest in using *A. mellifera* as a high-sensitivity bioindicator insect, because of their foraging activity and, consequently, their contact with pollutants present in the environment.

Hashimoto et al. [61] conducted various bioassays to detect changes in the activity on the esterase of Africanized *A. mellifera* after contamination by contact and by ingestion of the insecticide thiamethoxam neonicotinoid. In this study, it was found that five esterases (EST-1, EST-2, EST-3, EST-4, and EST-5) of these honeybees decreased in relative activity after infection with thiamethoxam, indicating that these isozymes exhibit a rapid response to poisoning by this neonicotinoid (Tables 2 and 3). Attencia et al. [62] evaluated the effects of parathion-methyl esterase in *A. mellifera* workers and found that, at a concentration of 0.01%, EST-1 activity was reduced by 75%, 14 and 21 days after the introduction of the insecticide. For esterases 3 and 4, there was 50% inhibition of its relative activity after 1-day release (Table 3). From these results, the authors suggested that inhibition of esterases 3 and 4 can be used to detect the presence of methyl-parathion residues in crops (Table 4).

Catae et al. [63] analyzed the effects of thiamethoxam in intestinal cells and Malpighian tubules in *A. mellifera* Africanized honeybees. Newly emerged workers were exposed up to 8 days with a diet containing sublethal doses of thiamethoxam LC_{50} equal to 1/10, i.e., 0.0428 ng a.i./L diet and found that the damage caused by thiamethoxam in the intestine was evident on bees exposed on the 1st day. Malpighian tubules presented abnormalities on the 8th day of exposure to the insecticide. Continuous exposure to sublethal doses of thiamethoxam can damage

organs that are used to metabolize the insecticide. *A. mellifera* Africanized honeybees newly emerged and exposed to a dose of 0.428 ng/day of thiamethoxam presented intoxication with sublethal dose of thiamethoxam, and Oliveira et al. [64] concluded that this can cause damage in the brains of bees.

	Ages/esterases																							
mg/mL	**Newly emerged**						**7 days**						**14 days**						**21 days**					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
2.0	+	−	−	+	+	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−
4.0	+	+	−	−	−	−	+	−	+	+	+	−	+	+	−	+	−	−	−	−	−	−	−	−
4.1	−	−	−	+	+	−	+	−	−	+	−	−	+	−	−	+	−	−	−	+	−	+	−	−
4.2	−	−	−	+	+	−	+	−	−	+	−	−	+	−	−	+	−	−	−	+	−	+	−	−
4.4	−	−	−	+	+	−	+	−	−	+	−	−	+	−	−	+	−	−	−	+	−	+	−	−

Modified from Ref. [61].

(+) = partial inhibition of esterase activity; (−) = absence of inhibition of esterase activity.

Table 2. Inhibition of relative activity of esterases detected in *A. mellifera* extracts of workers after contact with thiamethoxam

	Ages/esterases																							
mg/mL	**Newly emerged**						**7 days**						**14 days**						**21 days**					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
4.0	+	+	−	−	−	−	+	+	−	+	+	−	+	−	−	+	+	−	+	+	−	−	−	−
2.0	+	−	−	+	+	−	+	+	−	+	+	−	+	+	−	+	−	−	−	−	−	−	−	−
0.25	+	−	−	−	+	−	+	+	−	−	+	−	+	+	−	−	+	−	+	+	−	−	−	−
0.125	−	−	−	−	−	−	+	+	−	−	+	−	+	+	−	−	−	−	+	+	−	+	−	−
0.0625	−	−	−	−	−	−	+	+	−	−	+	−	+	+	−	−	+	−	+	+	−	+	−	−

Modified from [61].

(+) = partial inhibition of esterase activity; (−) = absence of inhibition of esterase activity.

Table 3. Inhibition of relative activity of esterases detected in extracts of *A. mellifera* workers after the application of thiamethoxam in the food

Studying *A. mellifera* honeybees, Yang et al. [65] noted that bees orally treated with imidacloprid insecticide were delayed in time to revisit a food source. This delay was dependent on the concentration of insecticide to which bees were exposed; at concentrations of 1,200 g/L many bees did not return to the food supply or to the colony. Rossi et al. [66] reported that low

doses of imidacloprid (0.809 ng/bee) caused cytotoxic effects on Malpighian tubules of Africanized *A. mellifera*.

		Esterase-1		Esterase-2	Esterase-3		Esterase-4	
Concentration	**Time (days)**	**Ac**	**But**	**But**	**Ac**	**But**	**Ac**	**But**
0.001%	1	*	*	*	*	*	*	*
	7	*	*	*	*	*	*	*
	14	*	*	*	*	*	*	*
	21	*	*	*	*	*	*	*
0.1%	1	*	*	*	--	*	*	-
	7	*	*	*	*	*	*	*
	14	---	*	+	*	+	*	+
	21	---	*	+	*	*	*	*
0.05%	1	---	-	-	---	-	---	-
	7	+	+	+	++	*	*	*
	14	*	*	*	*	*	-	*
	21	*	*	*	*	*	*	*
0.01%	1	*	*	*	*	*	*	*
	7	cd	cd	cd	cd	cd	cd	cd

Modified from Ref. [62].

+ =25% activity increase; ++ = 50% activity increase; - = 25% inhibition; -- = 50% inhibition; --- = 75% inhibition; * = no alteration; cd = colony death.

Table 4. Relative esterases activity in *A. mellifera* homogenates front to different concentrations of the methyl-parathion insecticide visualized with substrates 4-methylumbelliferyl acetate (Ac) and 4-methylumbelliferyl butyrate (But)

Kakamand et al. [56] indicated that deltamethrin, malathion, and thiamethoxam when orally administered to honeybees began to cause death of individuals after 4 hours, except for the deltamethrin 10-ppm dose, which began to kill honeybees 2 hours after the treatment. According to Kakamand et al. [56], the explanation of death after hours of poisoning is the difficulty of feeding and hydration of bees by paralysis of the digestive system or damage caused by insecticides to the intestine.

In a research conducted by Roat et al. [67], newly emerged bees and foragers from *A. mellifera* species were subjected to chronic treatment with fipronil insecticide at a dose of 0.01 ng (LD_{50}/100) for 3, 5, and 8 days, being subsequently subjected to cytochrome oxidase histochemical technique to assess the neural activity. Roat et al. [67] concluded that a basal marking in groups of newly emerged bees treated during 3 days similar to the control group, suggesting that neural activity in bees at this age is not altered by the presence of the insecticide, although symptoms of intoxication and changes in the survival of these individuals were noticed. A positive staining, most obvious, was observed only in the groups of forager bees treated during 3–5 days, causing changes in cell metabolism.

Arena and Sgolastra [68] compared the sensitivity of *A. mellifera* with 19 other species of bees by meta-analysis. Considering all species and pesticides evaluated, based on the LD_{50} and LC_{50}, Arena and Sgolastra [68] reported wide variation of the total radius of sensitivity measured between cases, and the statistics indicated *A. mellifera* is often more sensitive than other species. Nevertheless, considering the six chemical classes of insecticides used (carbamates, organochlorines, organophosphates, pyrethroid, neonicotinoids, and a mix of them), only for neonicotinoids all other species were more sensitive than *A. mellifera*. Hardstone and Scott [60] evaluated the susceptibility of *A. mellifera* and other insects, considering the same classes of insecticides, and pointed out that other species were more sensitive than *A. mellifera*.

Bees of genus *Tetragonisca* are rarely used in toxicity studies with insecticides. One of the studies of these bees was developed by Fermino et al. [69]. Species *Tetragonisca angustula* and *Tetragonisca fiebrigi* were used to evaluate the influence of nicosulfuron and paraquat herbicides on expression of isozyme esterase (EST – EC 3.1.1.1), malate dehydrogenase (MDH – EC 1.1.1.37), superoxide dismutase (SOD – EC 1.15. 1.1), soluble proteins, and brain cells by the critical electrolyte concentration technique (CEC).

Bioassays were performed by *in vitro* exposure to the herbicide in Petri dishes for 24 hours. Detected mortality was low being 2.75% for *T. angustula* and 5.8% for *T. fiebrigi* after contamination with nicosulfuron, and 2.5% for *T. angustula* and 1.25% for *T. fiebrigi* after contamination with paraquat (Table 5). However, changes in the expression of various isozymes and chromatin structure have been identified.

Authors verified that the herbicide nicosulfuron causes partial inhibition of esterases from *T. angustula* and *T. fiebrigi*. The herbicide paraquat promotes total inhibition of esterase relative activity in *T. fiebrigi* from the concentration of 1.5×10^{-5} g/mL, and in concentrations of 1.5×10^{-4} and 1.5×10^{-3} g/mL in *T. angustula*. Superoxide dismutase isozymes showed an increase in their relative activity after contamination with paraquat at 1.5×10^{-4} and 1.5×10^{-3} g/mL in both species. No changes were observed for MDH and soluble proteins. In the nerve cells, few changes were observed in gene expression after contact with the herbicides (Figures 1–4). According to the authors, Brazilian stingless bees *T. angustula* and *T. fiebrigi* have the potential to be used in biomonitoring for the presence of paraquat and nicosulfuron herbicides. *T. fiebrigi* has greater sensitivity to the herbicide paraquat than *T. angustula*.

Further studies are being developed by our research group with *T. angustula* and *T. fiebrigi* with insecticides fipronil, malathion, thiamethoxam, and growth regulators (neem and novaluron). Results obtained until now have shown that these stingless bees are susceptible to these insecticides and changes in the expression of esterases have been detected.

Bees *T. angustula* have esterases EST-3 and EST-4, whereas *T. fiebrigi* has EST-1, EST-2, and EST-4 [70]. Insecticide contamination promotes inhibition (decreased relative activity) of esterase EST-4 *Tetragonisca* bees, so this isozyme has the potential to become a bioindicator of environmental contamination with pesticides analyzed.

These studies have further shown that the *T. fiebrigi* bees are more susceptible to insecticides than *T. angustula* bees, so are more likely for population decline. A possible confirmation of this statement causes concern because *T. fiebrigi* bees have a more restricted distribution than

T. angustula bees. Camargo and Pedro [71] reported that the distribution of *T. fiebrigi* includes Argentina (Misiones, Tucumán); Bolivia (Santa Cruz); Brazil (Mato Grosso, Mato Grosso do Sul, Parana, Rio Grande do Sul, Brazil); Paraguay (Cordillera, Misiones). However, *T. angustula* is widely distributed in Americas: occurring from Mexico (Chiapas) to Brazil (in almost all regions of Brazil).

		T. angustula		*T. fiebrigi*	
Bioassay	**Concentration (g/mL)**	***N***	**Mortality (%)**	***N***	**Mortality (%)**
Control	----	56	0.0	62	0.0
Nicosulfuron	3.0×10^{-6}	60	0.0	63	0.0
	3.0×10^{-5}	57	6.5	79	1.25
	1.5×10^{-4}	60	0.0	73	8.75
	2.25×10^{-4}	46	8.0	55	9.83
	3.0×10^{-4}	60	0.0	54	10.0
Total		339	2.75	386	5.8
Control	----	60	0.0	40	0.0
Paraquat	1.5×10^{-6}	40	0.0	40	0.0
	1.5×10^{-5}	40	0.0	40	0.0
	1.5×10^{-4}	40	0.0	40	0.0
	1.5×10^{-3}	36	10.0	38	5.0
Total		216	2.5	198	1.25

Modified from Ref. [69].

N = number of bees analyzed.

Table 5. Mortality of *Tetragonisca angustula* and *Tetragonisca fiebrigi* in bioassays with the herbicides nicosulfuron (Sanson 40SC) and paraquat (Gramoxone 200) after 24-hour exposure

Other species of stingless bees have been evaluated for contamination with pesticides. Moraes et al. [5] evaluated the toxicity of some pesticides to *Scaptotrigona tubiba*. In the test of sprayed paper, delthamethrin, trichlorfon, and malathion showed an LC_{50} of 0.70, 0.26, and 0.015 ppm, respectively. *B. thuringiensis* had an LC_{50} higher than 336 ppm. For the topical application, delthamethrin, *B. thuringiensis*, and trichlorfon showed the respective LD_{50} of 0.73, 115.29, and 0.08 mg/bee. An LD_{50} higher than 0.04 mg/bee was inferred for malathion. All the insecticides were considered highly toxic by topical application route except *B. thuringiensis* that was relatively nontoxic.

Toxicity of pesticides by topical application in stingless bee species *Melipona beecheii, Trigona nigra,* and *Nannotrigona perilampoides* was performed by Valdovinos-Núñez et al. [72]. Results showed that for the three species, immature workers were more sensitive to pesticides than forager bees. These researchers also found that *M. beecheii* females were comparatively more resistant than males. However, queens were less resistant than the workers. Valdovinos-Núñez et al. [72] also assessed the toxicity of neonicotinoid insecticides for *N. perilampoides*. In this

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case, imidacloprid was more toxic than thiamethoxam and thiacloprid for stingless bee *N. perilampoides*.

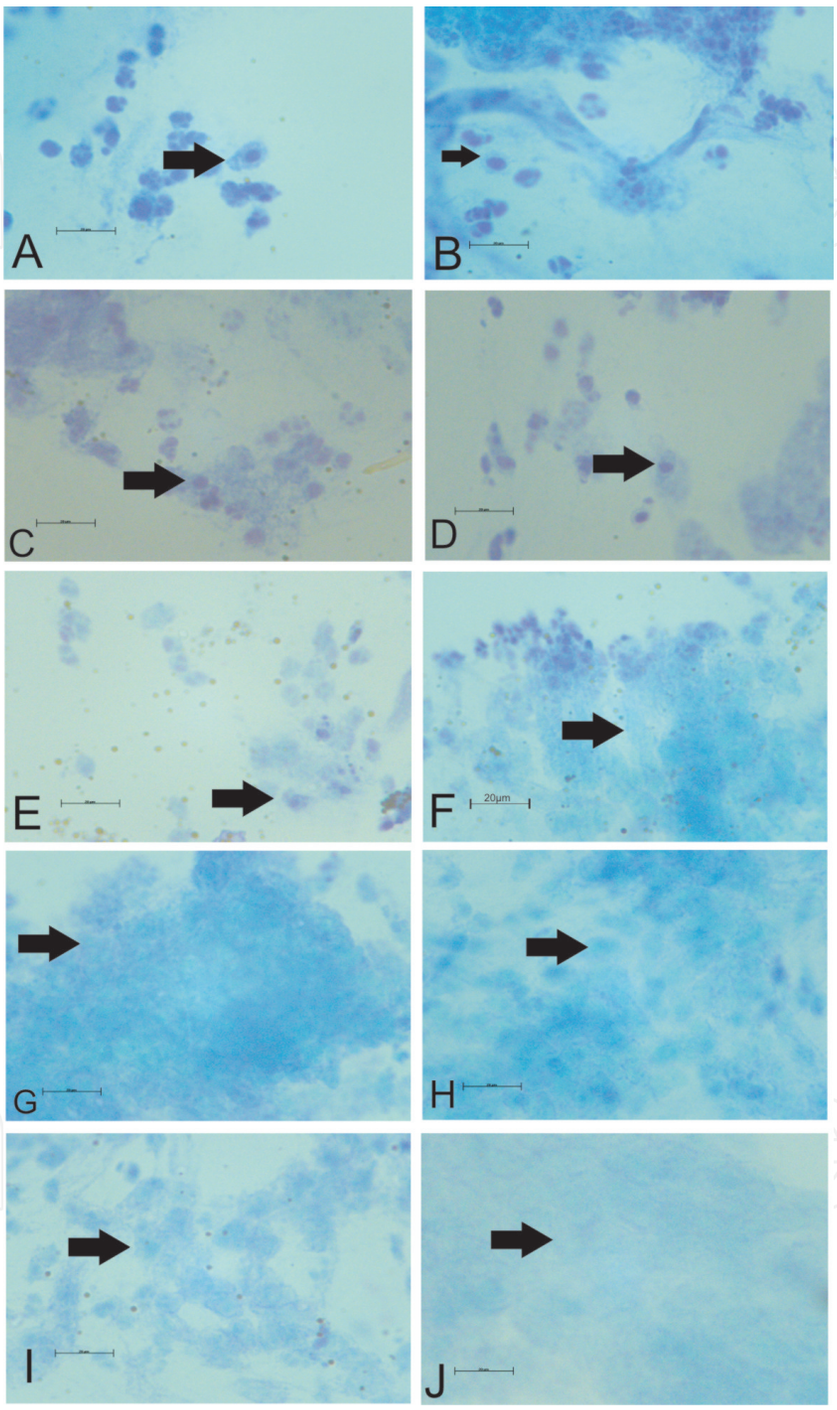

Figure 1. Critical electrolyte concentration analysis – nervous cells of *Tetragonisca angustula* after treatment with water (A–C–E–G–I) (Control), and paraquat 1.5×10^{-3} g/mL (B–D–F–H–J) stained with toluidine blue (TB) at pH 4.0 in the absence and presence of $MgCl_2$ (mol/L). A and B: TB without $MgCl_2$; C and D: TB + 0.05 mol/L $MgCl_2$; E and F: TB + 0.10 mol/L $MgCl_2$; G and H: 0.12 mol/L $MgCl_2$; I and J: TB + 0.15 mol/L $MgCl_2$. Arrows indicate the nuclei of neurons. Bar = 20 µm. Courtesy Fábio Fermino.

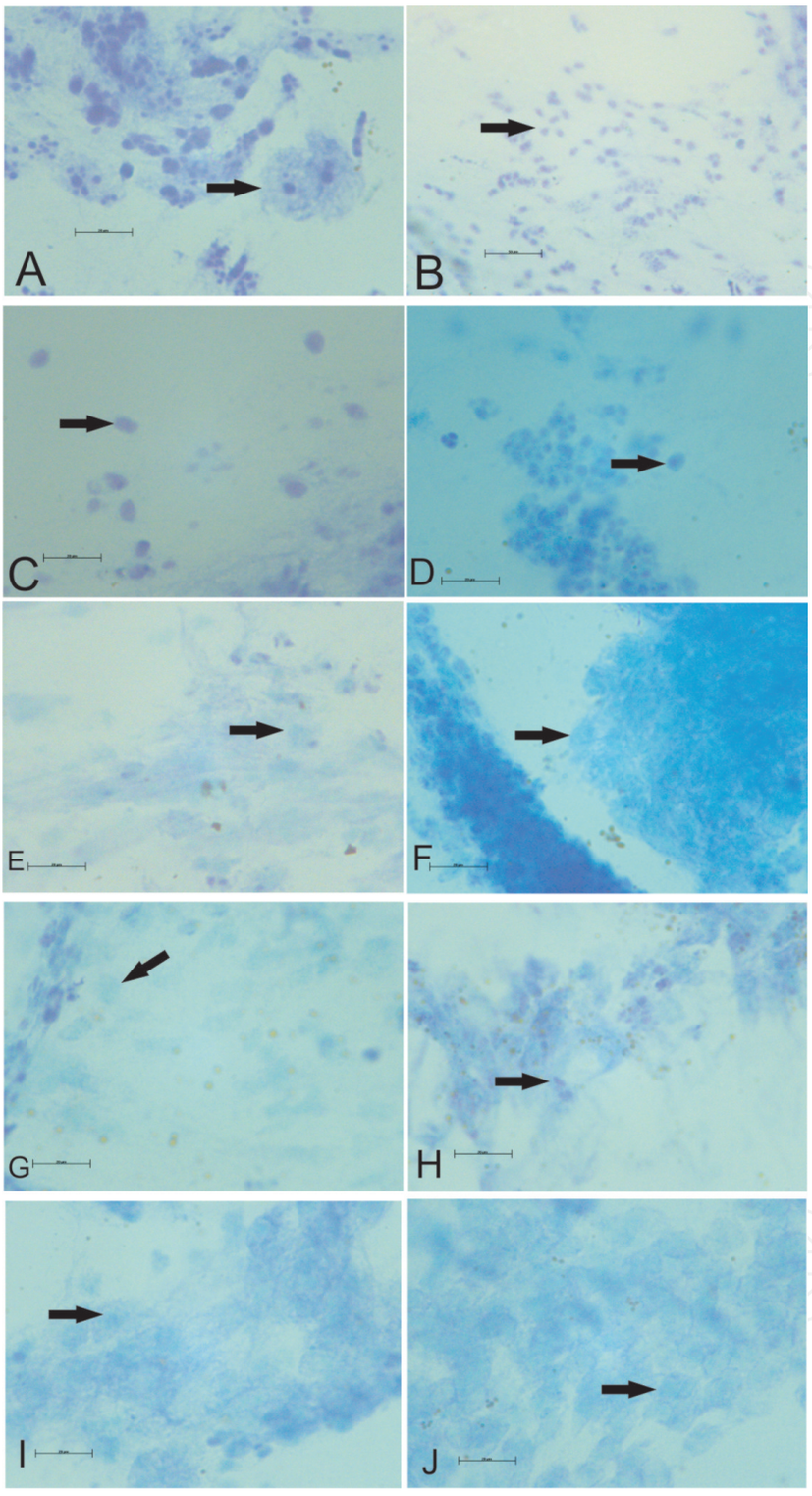

Figure 2. Critical electrolyte concentration analysis – nervous cells of *Tetragonisca fiebrigi* after treatment with water (A–C–E–G–I) (Control), and paraquat 1.5×10^{-3} g/mL (B–D–F–H–J) stained with toluidine blue (TB) at pH 4.0 in the absence and presence of $MgCl_2$ (mol/L). A and B: TB without $MgCl_2$; C and D: TB + 0.05 mol/L $MgCl_2$; E and F: TB + 0.10 mol/L $MgCl_2$; G and H: 0.12 mol/L $MgCl_2$; I and J: TB + 0.15 mol/L $MgCl_2$. Arrows indicate the nuclei of neurons. Bar = 50 μm. F = ommatidium presence in the lower left corner. Courtesy: Fábio Fermino.

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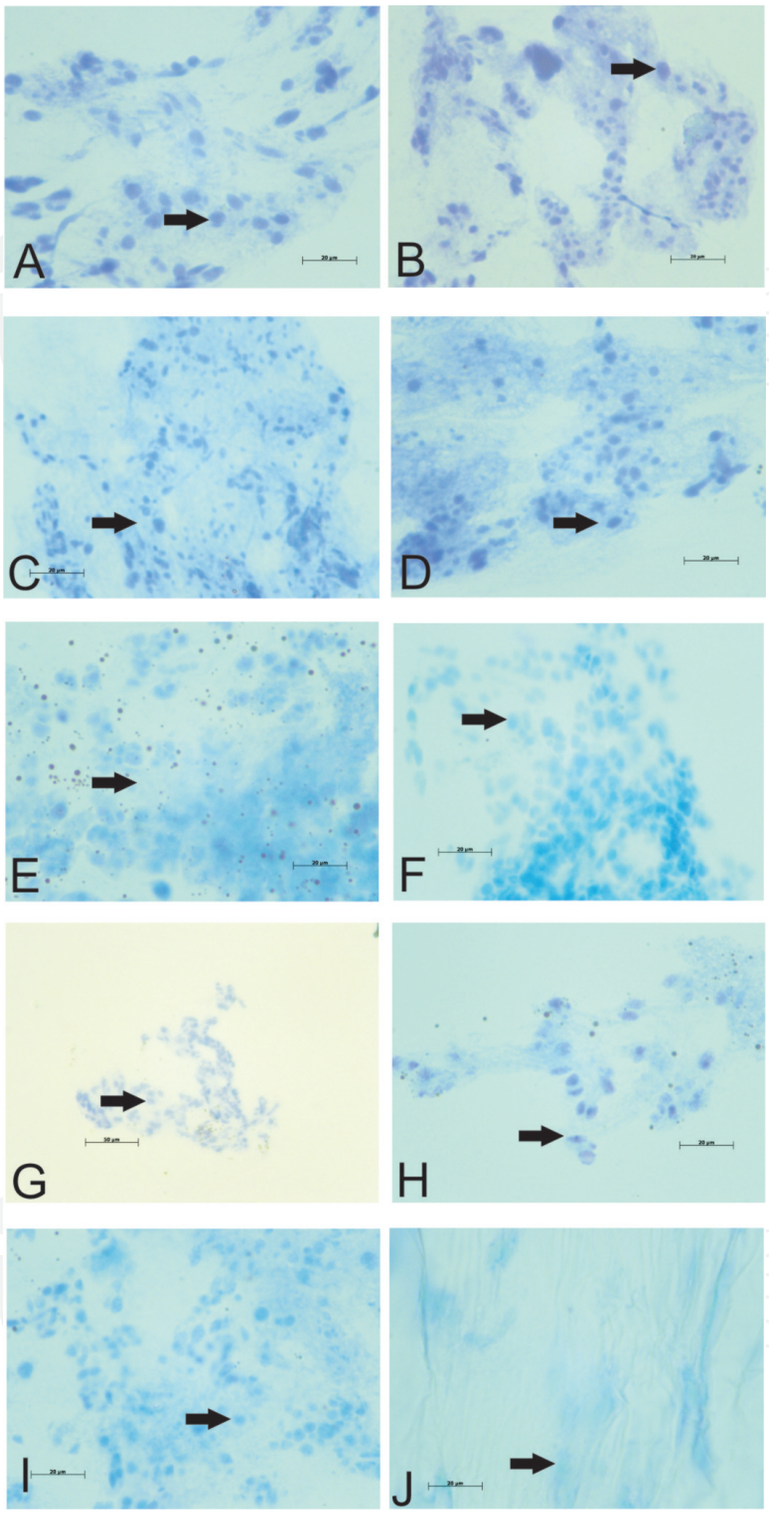

Figure 3. Critical electrolyte concentration analysis – nervous cells of *Tetragonisca angustula* after treatment with water (A–C–E–G–I) (Control), and nicosulfuron 3×10^{-4} g/mL (B–D–F–H–J) stained with toluidine blue (TB) at pH 4.0 in the absence and presence of $MgCl_2$ (mol/L). A and B: TB without $MgCl_2$; C and D: TB + 0.05 mol/L $MgCl_2$; E and F: TB + 0.10 mol/L $MgCl_2$; G and H: 0.15 mol/L $MgCl_2$; I and J: TB + 0.20 mol/L $MgCl_2$. Arrows indicate the nuclei of neurons. Bar = 20μm. Courtesy: Fábio Fermino.

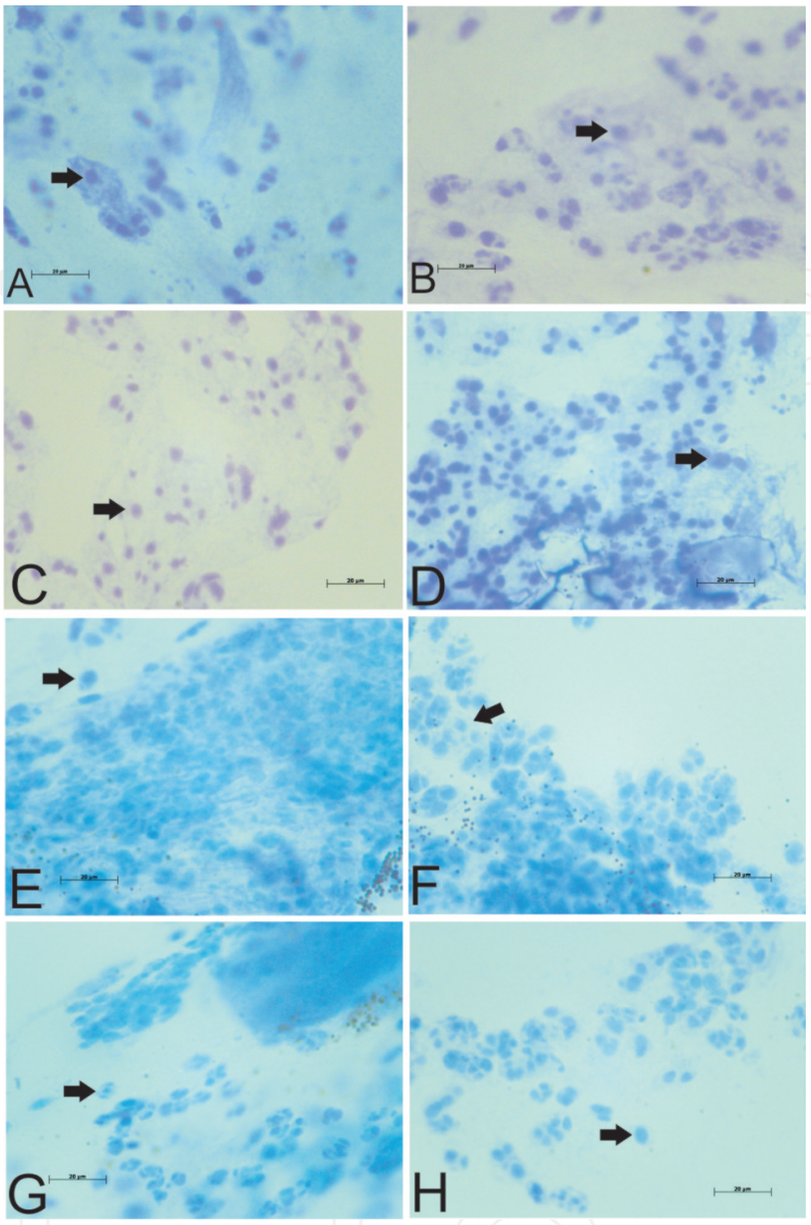

Figure 4. Critical electrolyte concentration analysis – nervous cells of *Tetragonisca fiebrigi* after treatment with water (A–C–E–G–I) (Control), and nicosulfuron 3×10^{-4} g/mL (B–D–F–H–J) stained with toluidine blue (TB) at pH 4.0 in the absence and presence of $MgCl_2$ (mol/L). A and B: TB without $MgCl_2$; C and D: TB + 0.05 mol/L $MgCl_2$; E and F: TB + 0.12 mol/L $MgCl_2$; G and H: 0.20 mol/L $MgCl_2$. Arrows indicate the nuclei of neurons. Bar = 20 μm. Courtesy: Fábio Fermino.

Considering fipronil insecticide, Jacob et al. [74] estimated the dose and lethal concentration (LD_{50} and LC_{50}) for stingless bees *Scaptotrigona postica*. Results of LD50 and LC50 obtained after 24-hour exposure were 0.54 ng a.i./bee and 0.24 ng a.i./L diet, respectively. These values are considered highly toxic to stingless bees. Contamination of *S. postica* with a diet containing fipronil (0.1 μg/kg) and boric acid 0.75% wt/wt presented a reduction in the survival [73]. These compounds caused changes in Malpighian tubules, which had dilatation of microvilli, ribosome loss of the rough endoplasmic reticulum, and an increase of the electron dense matrix of the mitochondria.

Azadirachtin (triterpenoid), found in neem oil (*Azadiractha indica*), is considered an *Insect Growth Regulator Insecticide* and causes inhibition of development, changing the metamorphosis of insects. This compound can be used as a natural insecticide with specific effects on different stages of growth of insects [75].

Commercial neem oil was evaluated in coffee crop in Apucarana region northwest of Paraná, Brazil (23° 33′5″ South, 51° 27′41″ West) in 2009, to verify changes in the relative activity of esterases on stingless bee *T. angustula* [76]. A bioassay was carried out in the field using *T. angustula* hive placed at 100 m of coffee crop (*Coffea arabica* L.) when the flower buds were beginning to open (Figure 5 A and 5 B); a hive 100 m from the coffee crop was used as a control and did not have an application of neem oil (in the same geographical region). Neem oil was prepared according to the manufacturer's instructions (100 mL of commercial product in 10 mL of water) and applied to the coffee culture; only water was used in the control. Analyses of esterase isozymes were performed with adult workers *T. angustula* collected after 24, 48, and 72 hours after application of neem oil. The control sample bees were collected in the same period.

In this study, it was observed that exposure of bees *T. angustula* to neem insecticide on the field after 48 hours of contamination led to a decrease in activity of the esterases EST-3 and EST-4, compared with the control (Figure 6). After 72 hours, the relative esterase activity was similar to the control (Figure 6). Evaluation of soluble proteins using denaturing sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) presented an increase in intensity of bands between 25 and 120 kDa after 24 hours of contamination. This was probably due to an increase in the synthesis of proteins, which act in detoxification of these bees. However, this method allowed identifying the displayed peptides (Figure 7).

Behavioral observations of *T. angustula* visitors of pulverized coffee flowers with neem oil showed that bees were repelled by visited flowers [76]. Use of the insecticide, neem, promotes changes in the expression of esterase isozymes and soluble proteins of *T. angustula*, but does not cause mortality of these bees. Effects on *T. angustula* nests will probably be observed in the long term and may damage the meliponiculture as an alternative source of income for small farmers.

The first reports about the disappearance of bees occurred in 2006, which alerted the scientific and nonscientific community about these insects, which perform an essential role in pollination plants, and as a consequence other species. The importance of bees for the environment is unquestionable, yet the contradiction between increasing the amount of food for the human population using chemicals for pest control and reducing the use of these compounds has generated discussions and further studies.

In Europe and United States, honeybees are extremely important in the pollination of crops and the decline of these pollinators has promoted a series of actions including governmental actions. One such action would be a ban on spraying aircraft that can carry pesticide residues for drifting to areas of forests or places with beekeeping and/or meliponiculture.

Brazil still uses pesticides that have been banned in the European Union and the United States. Reports of colony collapse disorder (CCD) occur in several regions. In addition to the Afri-

canized honeybees that occur in Brazil, there are over 400 species of stingless bees of unquestionable importance for pollination of native and cultivated plants. Furthermore, Brazilian meliponiculture has increased and the handling of many species has occurred.

Studies presented in the text show that stingless bees are susceptible to pesticides and may be contributing to the decline of their populations; however, we do not have estimates. The effects of the use of these pesticides have been described at the level of mortality and changes at the molecular level; we still need to conduct population studies to detect whether the decline can be attributed to pesticide use.

Despite the need for employment of pesticides on crops, it is extremely important that humanity find a balanced way to produce food and maintain the health of nature.

(A) (B)

Figure 5. A. View of coffee (*Coffea arabica*) crop flowering. B. View of *Tetragonisca angustula* nest entrance used in the bioassay in Apucarana, PR, Brazil. Courtesy: Mayra Cristina de Araujo.

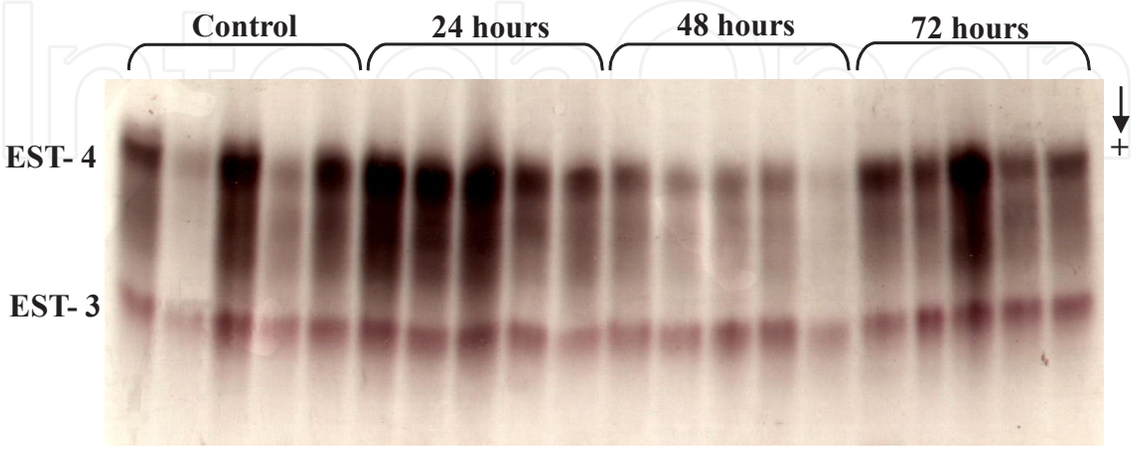

Figure 6. Electrophoresis profile of esterases in polyacrylamide gel electrophoresis (PAGE) of bee extracts *Tetragonisca angustula* after neem contamination. Courtesy: Mayra Cristina de Araujo.

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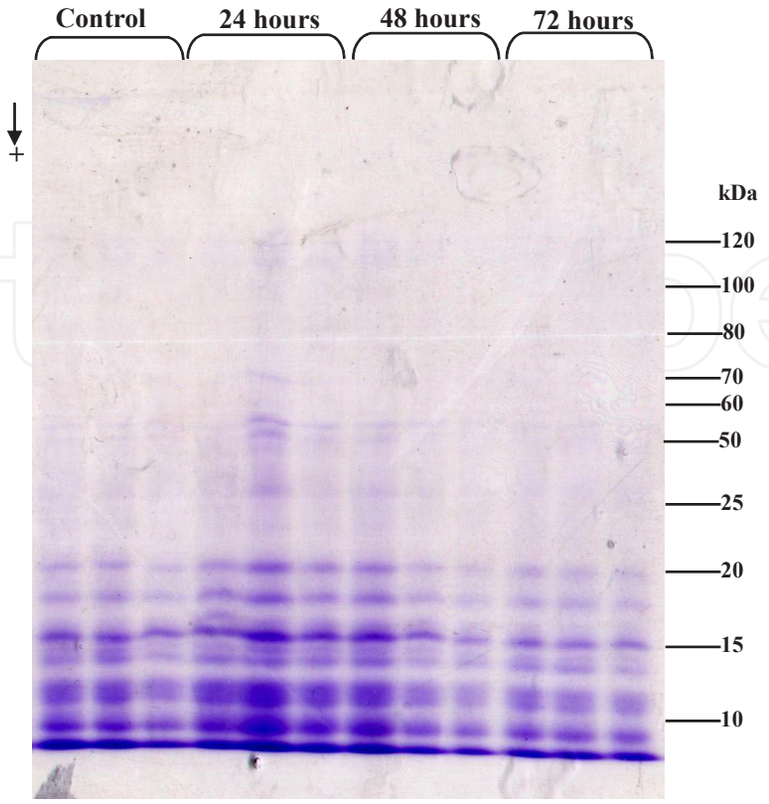

Figure 7. SDS-PAGE electrophoretic profile of *Tetragonisca angustula* extracts after neem contamination. Arrow indicates migration direction. KDa= molecular weight. Courtesy Mayra Cristina de Araujo

Acknowledgements

The authors thank CNPq (National Council for Scientific and Technological Development; Process 308283/2011-2, 482947/2013-6 and 311663/2014-1) for providing financial support. The authors also thank Prof. Dr. José Ricardo Penteado Falco for the orientation in the analysis of critical electrolyte concentration analysis and Juliana Mosconi Magro, MSc., for contributing to the text preparation.

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