Scientific Electronic Archives
Issue ID: Sci. Elec. Arch. Vol. 14 (2)
February 2021
DOI: http://dx.doi.org/10.36560/14220211292
Article link
http://sea.ufr.edu.br/index.php?journal=SEA&page=article&op=view&path%5B%5D=1292&path%5B%5D=pdf
Included in DOAJ, AGRIS, Latindex, Journal TOCs, CORE, Discoursio Open Science, Science Gate, GFAR, CIARDRING, Academic Journals Database and NTHRYS Technologies, Portal de Periódicos CAPES, CrossRef, ICI Journals Master List.

ISSN 2316-9281

Apis mellifera africanized queens tolerant to the neonicotinoid thiamethoxam

W. C. S. Pizzaia, N. C. Pereira, T. O. Diniz, V. A. A. Toledo, M. C. C. Ruvolo-Takasusuki

[1]Universidade Estadual de Maringá

*** Author for correspondence:** williambiologiaead@gmail.com

Abstract. Interaction between bees and plants ensured success in cross-pollination crops, increasing the vigor of the species, as the production of fruits and seeds. Bees are the main pollinators of angiosperms and several crops. Among the factors related to the disappearance of bees in agricultural areas is the excessive or improper use of insecticides. However, agriculture is extremely dependent on pesticides for pest control and the neonicotinoid thiamethoxam is one of the most used insecticides. The use of these chemicals affects the pollinating bee *Apis mellifera*. Thus, the aim of the study was to select Africanized *A. mellifera* queens tolerant to thiamethoxam, producing four generations of bees kept in chronic contamination and perform tests to validate tolerance. Colonies of *A. mellifera* were subjected to chronic contamination by contact with thiamethoxam at a sublethal concentration of 1.65 mg a.i./L. From the surviving colonies, four generations of new queens were produced, kept in chronic contamination with thiamethoxam. Chronic contamination caused changes in the workers' behavior for 25 days. Validation of selection and tolerance was performed in vitro bioassays to verify mortality and critical electrolyte concentration (CEC). In vitro bioassays showed that there was low mortality after contamination for 24 hours by contact with thiamethoxam at 82.5 mg a.i / L. The analyzes of CEC indicate that there was an increase in gene expression in brain cells, probably as an attempt to detoxify the workers' organism by contamination with thiamethoxam. New tests need to be carried out with colonies tolerant to the neonicotinoid thiamethoxam, but these initial results indicate that these queens have potential to tolerate this neonicotinoid.
Keywords: Genetic toxicology, apiculture, queen selection, mortality, thiamethoxam

Introduction

The interaction between bees and plants provided success in cross-pollination, which is a crucial evolutionary adaptation of plants (Couto e Couto, 2002; Stanley et al., 2017; Tong e Huang, 2017). Among the pollinating insects, *Apis mellifera* is one of the most important and perform most of the insect pollination. Honeybees diet includes pollen and nectar of various Angiosperms groups since it is a high generalist species. Furthermore, colonies of honeybees are numerous, reaching more than 100,000 individuals (Stein et al., 2017).

Despite the importance of pollination, agriculture is highly dependent on the use of agrochemicals. The reduction or absence of the use of these substances cause decline in agricultural production, increasing production costs and rising prices among other factors (Knutson, 1999; Pacífico-da-Silva et al., 2016; Lechenet et al., 2017; Ingram e Gemmill-Herren, 2018). Thus, insecticides, herbicides and fungicides are used to improve agricultural production.

The neonicotinoids are one of the most used insecticides, and it is efficient to control pests in crops. However, it can affect pollinators, especially bees. Bees contaminated with agrochemicals can lose their sense of direction and location, adding one more problem to CCD (Colony Collapse Disorder), which is a multifactorial disorder. The confusion arises from the fact that pesticides act on the nervous system of insects, promoting an excitement that can cause cramps and complications in communication, as it affects the waggle dance, either by blocking or by the release of neural receptor (Lu et al., 2012; Fairbrother et al., 2014; Laurino et al., 2013; Fischer et al., 2014, Lu et al., 2014; Hao et. al. 2016; Oliveira et al., 2018).

The neonicotinoid residues may be present in pollen and nectar of treated crops. Contact with this product has negative effects such as impairment of cognitive skills, including foraging, behavior and memory of foragers (Gontijo e Tice, 2003; Lu et al., 2012; Fairbrother et al., 2014; Sandrock et al., 2014;

Lu et al., 2014; Pacífico-da-Silva et al., 2016; Forfert et al., 2017; Oliveira et al., 2018).

Bees travel long distances to search for food. When they return to the colony, they use waggle dance to tell where the food is, passing the coordinates for other workers. This communication affects cognitive areas and contact with neonicotinoids can cause blockage in the synapses, stopping it from finding the colony after foraging (Carvalho et al., 2009; Fairbrother et al., 2014; Fischer et al., 2014; Lu et al., 2014; Pacífico-da-Silva et al., 2016).

The breeding of bees is an essential tool for apiculture development, and the exchange of queens and instrumental insemination is the basis for selecting and increasing genetic progress, mainly quantitative characteristics (Martinez et al., 2012; Zakour et al., 2014; Bourgeois et al.; 2016; Bienefeld, 2016).

Thus, this study aimed to select queen of Africanized *A. mellifera* tolerant to the neonicotinoid thiamethoxam, produce four generations of bees kept in chronic contamination with this insecticide and perform tests to validate tolerance.

Methods

Collection of bees

The collection of swarms of *A. mellifera* was made in Maringá, with geographic locations of latitude 23° 24' 34.8778" west longitude and 51° 56' 18.3272", and in neighboring cities. The collections, always in natural colonies, were made both in urban and rural areas, during the months of February, March and April 2015, in partnership with the fire department and city hall (Figure 1).

Figure 1. Material used for collection of *Apis mellifera* swarms for subsequent chronic contamination with 1.65 mg a.i/L of thiamethoxam.

Five swarms were collected for the experiment. The bees were placed in nucleus colonies, used as a nest along with the experiment. These colonies were kept in the apiary of the Experimental Farm of Iguatemi (FEI), belonging to the Universidade Estadual de Maringá.

Production of queen offspring

The swarm division technique was employed for the production of offprints of parental queens. Thus, a nucleus colony was placed over the other to expand the honeycomb. After the development of the cluster, nucleus colonies were separated and kept with five frames each, originating the next generation, and the existing nucleus colony remained with the old queen, and the new nucleus colony (next generation) remained with the honeycombs open offspring to stimulate the rise of a new queen.

The old nucleus colony was moved to a new location with the same queen and the new nucleus colony were at the same location of the former one, with the presence of forager bees, the development of queen cup and the birth of the new queen (Gupta et al., 2014).

Thiamethoxam Dilution

Bioassays were performed with thiamethoxam Analytical Standard (Fluka), diluted in acetonitrile at a concentration of 1.65 mg a.i./L. This concentration is indicated for thiamethoxam application in the soybean crop.

Chronic Contamination Bioassays

The bioassays were performed by contact, using filter paper with dimensions of 25 x 15 cm. The filter paper was soaked in a solution containing thiamethoxam 1.65 mg a.i./L, dried and then placed in the bottom of the nucleus colony. In control nucleus colonies the filter paper was soaked only in acetonitrile, dried and placed on the bottom of them (Figures 2 and 3). The exchange of filter papers was performed every 15 days throughout the experimental period.

Figure 2. Filter paper with 1.65 mg a.i/L thiamethoxam inserted into the nucleus colony.

After 45 days and one generation cycle, the production of queen offspring began and later mated naturally and introduced to a new nucleus colony for the next generation.

This process was carried out for four generations, aiming to verify tolerance to the insecticide thiamethoxam. The nuclei were labeled to follow the generations.

Field analysis

Throughout the experiment, which began in March 2015, observations were made on the behaviour of workers. The following characteristics were observed: changes in workers activity (care of offspring, constantly moving or still); the estimated number of bees; male offspring; mortality and colony abandonment (Figures 4 and 5); feeding; condition of the filter paper soaked in insecticide.

Toxicity Test

Toxicity tests were performed with adult workers, collected from the fourth generation of offspring, kept at the Experimental Farm Iguatemi-UEM. The workers were randomly collected within each colony nucleus. The workers collected from the nuclei colonies were placed in glass vials covered with voile tissue and transported to the laboratory of Animal Genetics at the Universidade Estadual de Maringá.

Figure 3. Nucleus colony identification.

Figure 4. *Apis mellifera* workers dead at the bottom of the nucleus colony after contamination by contact with 1.65 mg a.i./L of thiamethoxam.

The bioassays were performed with thiamethoxam at a concentration of 82.5 mg a.i./L. The experiments were executed with three replicates and one control. Twenty workers were placed in each glass vial. Contamination by contact was performed with filter paper soaked with 1mL of thiamethoxam and allowed to dry before being placed on the bottom of vials. In the control group, the filter paper was embedded in acetonitrile (solvent of active ingredient thiamethoxam). The experiment was performed for a period of 24 hours in BOD incubator (Biological Oxygen Demand) with a temperature of 33 ± 2°C and relative humidity of 80 ± 10%. The control was maintained under the same conditions but in separate BOD.

Figure 5. Nucleus colony abandoned after chronic contamination with 1.65 mg a.i./L of thiamethoxam.

Glass vials also contained a water-soaked cotton swab and a container with food (candy). The entry of the glass vials was closed with voile fabric. During the experiments, bees were movement and flighting inside the glass vials. At the end of the bioassay, mortality was estimated.

Critical Electrolyte Concentration (CEC)

The filter paper inside the nuclei over the generations allowed a contact simulation with thiamethoxam, as if the bees had contact with it in the field, considering the evaluation of behavior and cytochemical analysis, honeybee samples were collected from the Experimental Farm of Iguatemi maintained in contact with the neonicotinoid thiamethoxam at a concentration of 1.65 mg a.i./L.

To investigate changes in the brain and hypopharyngeal gland chromatin structure, the protocol described by Vidal and Mello was used (1989). The tissues bees were dissected, placed in saline solution for insects (NaCl 0.1 M, Na2HPO4 0.1 M and KH2PO4 0.1 M), extended in microscopy slides, with acetic acid (45%) and crushed under a glass slide. Microscopy slides were frozen in liquid nitrogen and the glass slide removed when it reached room temperature. The material was fixed in ethanol:acetic (3:1 v/v) acid for 1 min and the slide was washed in ethanol for 5 min.

The tissues were stained for 20 min with TB 0.025% in a McIlvaine buffer (pH 4.0), contained different MgCl2 concentrations (0.0; 0.02; 0.05; 0.08; 0.10; 0.12; 0.15; 0.20; and 0.30 mol/L). Then, slides were washed in distilled water and air-dried, bleached in Xylol for 15 min, assembled in Entellan, analyzed and photographed under a Zeiss standard optical microscopy. The cell nuclei stained violet were the controls and the green color corresponding to the CEC point.

Results and discussion

Production tolerant queens to thiamethoxam

From the five swarms collected in nature four generations were made for each colony during 2015. The F1 generation started in April / 2015; F2 in August / 2015 F3 in October / 2015 and F4 in December / 2015.

The swarm division technique was employed to produce the queen offspring, thus creating the generations F1, F2, F3 and F4. When the queen was laying eggs, the nucleus was separated, and a new generation started. There was a considerable production of queen cells for new queens, which ensured the nucleus colony development (Figures 6, 7 and 8).

Figure 6. Storage of honey, pollen and brood cells in colony nucleus with chronic contamination with 1.65 mg a.i./L of thiamethoxam.

Artificial multiplication of colonies is a beekeeping technique to maintain the genetic material, thereby multiplying the existing positive characteristics in a group. It also allows the quality of the lineage of the colonies, selecting specific features, either for production or tolerance to pests and diseases (Campos, 2008; Martinez et al, 2012).

Bees over the generations had a continuous hygienic behavior, cleaning the nucleus of the colony, keeping in touch with the filter paper containing thiamethoxam. In the analyzed generations, the bees always kept their forage activities, storage, production of honey, honeycomb production, the royal jelly and expel the hive of drones during the winter.

After the first generation, the behavior changes were not observed anymore and the queens produced normal eggs. Although some behavior changes at the beginning of the generation F1 on the nucleus colonies A and B were observed, with honey storage in central honeycombs.

Neonicotinoid thiamethoxam was initially very toxic, affecting the physiology and behavior of bees, even in small concentrations, confirming the suggestion of many researchers that the insecticides are jeopardizing the development of bees, contributing to its disappearance in the fields, known as colony collapse disorder (CCD). The use of insecticide has been one of the main causes of this multifactorial disorder

Figure 7. Observation of offspring in nucleus colony after contamination with 1.65 mg a.i./L of thiamethoxam.

.

Figure 8. Construction of queen cells by workers in nucleus colony with chronic contamination with 1.65 mg a.i/L of thiamethoxam.

Obtaining queens tolerant to insecticides is a collaboration for beekeeping because selecting queens with this feature, combined with other characteristics, such as resistance to pests or parasites, guarantees the beekeeper more productive colonies, both for resistance to factors that threaten the survival bee and for production.

The characteristic of tolerance to the insecticide can assure colony protection because when the bee collects nectar and pollen of plants with insecticides residues in sublethal doses, it returns to the colony and indicates the place for food collection. Therefore, tolerant bees avoid CCD.

A young queen is a sign of high production and high honey quality, which, coupled with the selection of tolerance for insecticides and disease, can improve the production of honey, pollen and wax, also reducing the use of other protection products for apiaries.

Honeybees behavior following chronic contamination with thiamethoxam

The bees grooming behavior was observed in the filter paper, that was partial or total removed (Figure 9), ensuring contact with the insecticide. At the beginning of the contamination, bees showed locomotion difficulty (walking and flying), tremors and spasms, as well as changes in behavior characteristics such as pollen storage, honey and propolis.

These changes were visible during maintenance of the colonies, but after the emergence of new workers and drones, behavior and activities were resumed, and the provided food was always consumed (Figure 6).

Figure 9. Filter paper containing 1.65 mg a.i/L thiamethoxam removed by the workers of *Apis mellifera*.

The hygienic behavior characterized by the internal service of the workers includes cleaning the hive, unsealed the cells of the honeycomb and remove dead or contaminated offspring among other content in the colony. Ensuring the population dynamics of the species which gives a barrier to prevent the development of diseases or parasites (Arathi et al., 2000).

Although the location of the apiary showed favorable conditions for the proper development of the colonies, after nine months of chronic treatment with thiamethoxam, two parental colonies left the hives, although it was good condition.

The collection of nectar always occurred so that the nucleus colonies were always with food stock for the swarm. The nucleus colony consists of five wooden frames where the honeycombs are inserted. The central frame is where the queen bee is laying eggs and side frames are where the bees deposit honey in the combs. Bees of some nuclei had an important characteristic behavior: deposit honey center of the nucleus colony even with the presence of the queen and the holding of egg laying

The hygienic behavior of workers involves cleaning the hive, unsealed the cells of the honeycomb and remove dead or contaminated offspring among other contents present in the colony. This ensures the population dynamics of the species which prevents the development of diseases or parasites (Arathi et al., 2000; Bienefeld et al., 2016; Leclercq et al., 2017; Cheruiyot et al., 2018; Leclercq et al., 2018).

A. mellifera is a highly social insect, which contributes to its adaptability to the environment, giving it its characteristics, as offspring care, allowing overlapping generations and ensuring the maintenance of the colony, even with a population size on average from 50,000 to 80,000 individuals. Bees rely on raising stocks and ensure the survival of the colony, and working together is necessary in an orderly and efficient manner, ensuring the survival and future of the colony (Michener, 1974; McFarland, 1993; Bianchini e Bedendo, 1998).

The neonicotinoids are used to control insect pests in crops, but their residues in nectar may directly affect the bees, changing their physiology and cognitive skills, including foraging, behavior and memory (Fischer et al, 2014; Sandrock et al, 2014), as observed in chronic treatment with thiamethoxam 1.65 g a.i./L.

Validation of colonies tolerant to thiamethoxam

Thiamethoxam toxicity in F4 generation

The acute contamination performed in vitro showed reduced mortality of workers F3 and F4 when compared with the control F3. The lowest survival was observed in F3 A (86.67%) and the highest survival in F4B and F3C showed the same survival rates (88.33%) (Table 1).

Table 1. In vitro toxicity test by contact for 24 hours with thiamethoxam 82.5 mg a.i/L in F3 generations nucleus colony A, F4 nucleus colony B and F3 nucleus colony C. Control F3 was contaminated by contact with acetonitrile

Generation/Repetition	1	2	3	Survival (%)
Control F3	19	20	20	98.33
F3 A	18	17	17	86.67
F4 B	17	19	17	88,33
F3 C	17	18	18	88.33

During the bioassays, bees destroyed the filter paper, which was in the bottom of the container, containing the thiamethoxam solution, the same behavior was observed in the field, regarding food and water consumption and grouping to maintain the temperature.

Selected queens from the F4 generation showed tolerance to thiamethoxam but still cannot be considered tolerant. For this, the number of generations needs to be higher to obtain a lineage of queens tolerant to that neonicotinoid.

The neonicotinoids are toxic and can cause harmful damage to the central nervous system, causing tremors, difficulty in movement, excitability and ultimately death (Pisa et al., 2017).

Several studies seek to understand the damage caused by insecticides, to unravel the action of these products on the lives of insects, because they act directly and systematically, impairing several bee behaviors (Blaquière et al., 2012; Pisa et al., 2017; Wood et al., 2018). Selected queens of F4 generation showed tolerance to thiamethoxam.

Critical Electrolyte Concentration (CEC)

The cytochemical analysis showed that the brain CEC point of the control group was at the concentration of 0.30%, while in the F3 generation occurred at the concentration of 0.15%, indicating a decrease in chromatin condensation. It can be inferred that there were chromatin de-condensation and consequent increase in gene expression (Table 2). In the F4B and F3C nucleus colony, there was an increase in the chromatin condensation of brain cells, but the values could not be discerned due to the methodology employed. Chromatin condensation indicates that there was a decrease in gene expression in these two nuclei (Table 2).

In the hypopharyngeal gland of workers from the control group, it was not possible to determine the point of CEC in the analyzed concentrations, but the F4B colony and the F3C colony, the CEC points were 0.20% and 0.30%, showing that there were a decrease in chromatin condensation and consequent increase in gene expression (Table 2).

DNA not complexed with protein and DNA-protein complexes in chromatin somatic cells usually exhibit basophilia metachromatic in violet color when stained with toluidine blue solution at pH 3.6 - 4.0 (Mello, 1997; Sridharan e Shankar, 2012). With toluidine blue in the presence of Mg^{2+} ions occur a dispute to the connection point or negative charges of the DNA or RNA and metachromasia disappears in an assigned concentration of Mg^{2+} ion (Mello, 1997; Sridharan e Shankar, 2012).

CEC results showed that after chronic contamination over four generations of *A. mellifera* nucleus colony, different responses to pesticide tolerance was observed (increase and decrease of gene expression), which probably alters both physiology and development of workers. According to Christen et al. (2018), all neonicotinoids led to significant alteration (mainly down-regulation) of gene expression, generally with a concentration-dependent effect.

The results showed that the offspring of tolerant queens (F4) showed gene expression alteration, probably due to selection for neonicotinoid tolerance. Christen et al. (2018) concluded in the study of *A. mellifera* contaminated with three neonicotinoids, including thiamethoxam, that genes related to metabolism and detoxification were differently expressed.

Thus, these authors showed that neonicotinoids led to transcriptional alterations, especially down-regulation, of brain genes from honeybee workers. Besides, genes involved in sulfur metabolism were up-regulated, probably due to the increased need for glutathione, which plays a role in the detoxification of reactive oxygen species.

Table 2. Nuclear basophilia of brain and hypopharyngeal glands (HG) of workers from *Apis mellifera* generations F3 and F4, exposed to chronic contamination with thiamethoxam 1.65 mg a.i./L, stained with toluidine blue 0.025% (TB) added with $MgCl_2$ in various concentrations (mol/L)

	Control		F 3 A		F4 B		F3 C	
	Brain	HG	Brain	HG	Brain	HG	Brain	HG
AT lacking $MgCl_2$	Vi	Vi	Vi	Vi	Vi	Vi	Vi	Vi
AT + $MgCl_2$ 0,02%	Vi	Vi	Vi	Vi/ Bl	Vi	Vi	Vi	Vi
AT + $MgCl_2$ 0,05%	Vi/ Bl	Vi/ Bl	Vi/ Bl	Vi/ Bl	Vi	Vi	Vi/ Bl	Vi/ Bl
AT + $MgCl_2$ 0,08%	Vi/Bl	Vi/Bl	Vi	Vi	Vi	Vi	Vi/ Bl	Vi/ Bl
AT + $MgCl_2$ 0,10%	Vi	Vi	Vi	Vi/ Bl	Vi/ Bl	Vi/Bl	Vi/Bl	Vi
AT + $MgCl_2$ 0,12%	Bl/Gr	Bl/Gr	Vi/Bl	Bl/Gr	Vi/Bl	Bl/Gr	Vi/Bl	Bl/Gr
AT + $MgCl_2$ 0,15%	Vi/Bl	Vi/Bl	Gr	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl
AT + $MgCl_2$ 0,20%	Vi	Vi	Gr	Bl/Gr	Vi/Bl	Gr	Vi/Bl	Vi/Bl
AT + $MgCl_2$ 0,30%	Gr	Vi/Bl	Vi/Bl	Vi/Bl	Vi/Bl	Gr	Bl/Gr	Gr
CEC Value	0,30	--------	0,15	--------	--------	0,20	---------	0,30

Vi = violet; Bl = blue; Gr = green

Alteration in gene expression related to nerve cells and development of *A. mellifera* was studied by Tavares et al. (2019), analysing pupae and newly emerged *A. mellifera* workers submitted to sublethal concentrations of thiamethoxam. After the contamination, the mushroom bodies and the antennal lobes were analyzed, because these brain structures are involved in stimuli reception, learning and memory consolidation. With the results, the authors inferred that exposure to sublethal concentrations of thiamethoxam during the larval stage might cause neurophysiological disorders in honeybees.

Although our study is initial, chronic contamination in sublethal concentrations with the neonicotinoid thiamethoxam likely allows bees to develop different forms of tolerance to this pesticide. However, further studies are needed to identify which genes are related to the morphological, physiological, behavioral and developmental changes that may have occurred for tolerance.

Conclusion

Chronic treatment with thiamethoxam at a concentration of 1.65 mg a.i./L initially caused behavioral changes in the workers. After 25 days of chronic treatment, the nucleus colonies resume their normal activities. Toxicology tests and CEC showed that selected queens maintained on chronic were tolerant of this neonicotinoid.

References

ARATHI, H.S., BURNS, I., SPIVAK, M. Ethology of hygienic behaviour in the honey bee, *Apis mellifera* L. (Hymenoptera: Apidae): behavioural repertoire of hygienic bees. Journal of Ethology, Vol. 106, p. 365-379, 2000.

BIENEFELD, K. Breeding Success or Genetic Diversity in Honey Bees? Bee World, Vol. 93, p. 40-44, 2016.

BIENEFELD, K., ZAUTKE, F., GUPTA, P. A novel method for undisturbed long-term observation of honey bee (*Apis mellifera*) behavior – illustrated by hygienic behavior towards varroa infestation. Journal of Apicultural Research, Vol. 54, p. 541-547, 2016.

BIANCHINI, L., BEDENDO, I.P. Efeito antibiótico da própolis sobre bactérias fitopatogênicas. Scientia Agricola, Vol. 55, p. 149-152, 1998.

BLAQUIÈRE, T., SMAGGHE, G., VAN-GESTEL, C. A., MOMMAERTS, V. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. Ecotoxicology, Vol. 21, p. 973-992, 2012.

BOURGEOIS, A.L., RINDERER, T., GUZMAN, L.I., HOLLOWAY, B. Molecular genetic analysis of Varroa destructor mites in brood, fallen injured mites, and worker bee longevity in honey bees. Journal of Apicultural Research, Vol. 54, p. 328-334, 2016.

CAMPOS, J.C.P. Melhoramento genético aplicado à produção animal. Belo Horizonte - MG: FEPMVZ, 2008. 617p.

CARVALHO, S.M., CARVALHO, G.A., CARVALHO, C.F., BUENO-FILHO, J.S.S., BAPTISTA, A.P.M. Toxicidade de acaricidas/inseticidas empregados na citricultura para a abelha africanizada *Apis mellifera* L., 1758 (hymenoptera: apidae). Arquivos do Instituto Biológico, Vol. 76, p. 597-606, 2009.

CHERUIYOT, S.K., LATTORFF, H.M.G., KAHUTHIA-GATHU, R., MBUGI, J.P., MULI, E. Varroa-specific hygienic behavior of *Apis mellifera scutellata* in Kenya. Apidologie, Vol. 49, p. 439-449, 2018.

COUTO, R.H.N., COUTO, L.A. Apicultura: manejo e produtos. Jaboticabal: FUNEP, 2002. 191p.

CHRISTEN, V., SCHIRRMANN, M., JUERG, E.F., KARL, F. Global Transcriptomic Effects of Environmentally Relevant Concentrations of the Neonicotinoids Clothianidin, Imidacloprid, and Thiamethoxam in the Brain of Honey Bees (*Apis mellifera*). Environmental Science Technology, Vol. 52, p. 7534-7544, 2018.

FAIRBROTHER, A., PURDY, J., ANDERSON, T., FELL, R. Risks of neonicotinoid insecticides to honeybees. Environmental Toxicology and Chemistry, Vol. 33, p. 719-731, 2014.

FISCHER, J., MULLER, T., SPATZ, A.K., GREGGERS, U., GRUNEWALD, B., MENZEL, R. Neonicotinoids interfere whit specific components of navigation in honeybees. Plos One, Vol. 9, p. 1-10, 2014.

FORFERT, N., TROXLER, A., RETSCHNIG, G., GAUTHIER, L., STRAUB, L., MORITZ, R.F.A., NEUMAN, P., WILLIAMS, G.R. Neonicotinoid pesticides can reduce honeybee colony genetic diversity. Plos One, Vol. 12, p. 1-14, 2017.

GONTIJO, A.M.M.C., TICE, R. Teste do cometa para a detecção de dano no DNA e reparo em células individualizadas. In: RIBEIRO, L.R., SALVADORI, D.M.F., MARQUES, E.K. Mutagênese Ambiental. Canoas: Ulbra, 2003. p. 173-200

GUPTA, R.K., REYBROECK, W., LAGET, D., EERENS, J., LANDSHEERE, P., PAUW, M. Techniques in Beekeeping. In: GUPTA, R.; REYBROECK, W.; VAN VEEN, J.; GUPTA, A. (eds) Beekeeping for Poverty Alleviation and Livelihood Security. Springer: Dordrecht, 2014. p.557-597.

HAO, Y., LI, J. Proteomic Research on Honeybee Diseases. In: SALEKDEH, G. (eds). Agricultural Proteomics. Springer, 2016. p. 289-298.

INGRAM, D.S., GEMMILL-HERREN, B. Pollination services to agriculture: sustaining and enhancing a key ecosystem service. Food Security, Vol. 10, p. 1669-1672, 2018.

KNUTSON, R.D. Economic impacts of reduced pesticide use in the United States: measurement of costs and benefits. Agricultural and Food Policy Center, 1999. 26p

LAURINO, D., MANINO, A., PATETTA, A., PORPORATO, M. Toxicity of neonicotinoid insecticides on different honey bee genotypes. Bulletin of Insectology, Vol. 66, p. 119-126, 2013.

LECHENET, M., DESSAINT, F., PY, G., MAKOWSKI, D., MUNIER-JOLAIN, N. Reducing pesticide use while preserving crop productivity and profitability on arable farms. Nature Plants, Vol. 3, p. 1-6, 2017.

LECLERCQ, G., FRANCIS, F., GENGLER, N., BLACQUIÈRE, T. Bioassays to Quantify Hygienic Behavior in Honey Bee (*Apis mellifera* L.) Colonies: A Review. Journal of Apicultural Research, Vol. 57, p. 663-673, 2018.

LECLERCQ, G., PANNEBAKKER, B., GENGLER, N., NGUYEN, B. K., FRANCIS, F. Drawbacks and benefits of hygienic behavior in honey bees (*Apis mellifera* L.), a review. Journal of Apicultural Research, Vol. 56, p. 366-375, 2017.

LU, C., WARCHOL, K.M., CALLAHAN, R.A. Sub-lethal exposure to neonicotinoids impaired honey bees winterization before proceeding to colony collapse disorder. Bulletin of Insectology, Vol. 67, p. 125-130, 2014.

LU, C., WARCHOL, K.M., CALLAHAN, R.A. In situ replication of honey bee colony collapse disorder. Bulletin of Insectology, Vol. 65, p. 99-106, 2012.

MARTINEZ, O.A., SOARES, A.E.E. Melhoramento genético na apicultura comercial para produção da própolis. Revista Brasileira de Saúde e Produção Animal, Vol. 13, p. 982-990, 2012.

MCFARLAND, D. The complex behaviour of honeybees. In: LONGMAN, A.W. Animal behaviour: psychobiology, ethology and evolution. Oxford: University of Oxford Press,1993. p. 441-461.

MELLO, M.L.S. Cytochemistry of DNA, RNA and nuclear proteins. Brazilian Journal Genetics, Vol. 20, p. 257-264, 1997.

MICHENER, C.D. The social behavior of the bees: a comparative study. Cambridge, Massachusetts: The belknap press of harvard university press, 1974.

OLIVEIRA, A.C., JUNQUEIRA, C.N., AUGUSTO, S.C. Pesticides affect pollinator abundance and productivity of sunflower (*Helianthus annuus* L.), Journal of Apicultural Research, Vol. 58, p. 2-8, 2018.

PACÍFICO-DA-SILVA, I., MELO, M.M., BLANCO, B.S. Efeitos tóxicos dos praguicidas para abelhas. Revista Brasileira de Higiene e Saúde Animal, Vol. 10, p. 142-157, 2016.

PISA, L., GOULSON, D., YANG, E.C., GIBBONS, D., SÁNCHEZ-BAYO, F., MITCHELL, E., AEBI, A., SLUIJS, J., MACQUARRIE, C.J.K., GIORIO, C., LONG, E.Y., MCFIELD, M., LEXMOND, M.B., BONMATIN, J.M. An update of the Worldwide Integrated Assessment (WIA) on systemic insecticides. Part 2: impacts on organisms and ecosystems. Environmental Science and Pollution Research, Vol. 24, p.1-49, 2017.

SANDROCK, C., TANADINI, M., TANADINI, L.G., FAUSER-MISSLIN, A., POTTS, S.G. Impact of Chronic Neonicotinoid Exposure on Honeybee Colony Performance and Queen Supersedure. Plos One, Vol. 9, p. 1-13, 2014.

SRIDHARAN, G., SHANKAR, A.A. Toluidine blue: A review of its chemistry and clinical utility. Journal of Oral Maxillofacial Pathology, Vol. 16, p. 251-255, 2012.

STANLEY, J., SAH, K., SUBBANNA, A.R.N.S. How efficient is the Asian honey bee, *Apis cerana* in pollinating mustard, *Brassica campestris* var. toria? Pollination behavior, pollinator efficiency, pollinator requirements and impact of pollination. Journal of Apicultural Research, Vol. 56, p. 439-451, 2017.

STEIN, K. COULIBALY, D., STENCHLY, K., GOETZE, D., POREMBSKI, S., LINDNER, A., KONATÉ, S., LINSENMAIR, E.K. Bee pollination increases yield quantity and quality of cash crops in Burkina Faso, West Africa. Scientific Reports, Vol. 7, 17691, 2017.

TAVARES, D.A., ROAT, T.C., SILVA-ZACARIN, C.M., NOCELLI, R.C.F., MALASPINA, O. Exposure to thiamethoxam during the larval phase affects synapsin levels in the brain of the honey bee. Ecotoxicology and Environmental Safety, Vol. 169, p. 523-528, 2019.

TONG, Z.Y., HUANG, S.Q. Safe sites of pollen placement: a conflict of interest between plants and bees? Oecologia, Vol. 186, p. 163-171, 2017.

WOOD, S.C., KOZI, I.I.V., KOZIV, R.V., EPP, T., SIMKO, E. Comparative chronic toxicity of three neonicotinoids on New Zealand packaged honey bees. PLoS ONE, Vol. 13, p. 1-19, 2018.

ZAKOUR, K.M, BIENEFELD, K. Basic considerations in the development of breeding plans for honey bees, illustrated by data on the native Syrian honey bee (*Apis mellifera syriaca*), Journal of Apicultural Research, Vol. 53, p. 314-326, 2014.