FLORA AND FAUNA
DOI:10.33451/florafauna.v23i1pp237-234
2017 Vol. 23 No. 1 PP 237-244
ISSN 0971 - 6920

STUDY OF ACETYL CHOLINESTERASE ACTIVITY IN FORAGER WORKER BEES OF *APIS MELLIFERA* UNDER STRESS OF PESTICIDES

SUSHIL KUMAR

Department of Zoology,
Govt. PG College, BISALPUR,
PILIBHIT-262201 (U.P.) INDIA
Email: sushilsoni021@gmail.com

Received : 27.02.2017; ***Accepted*** : 25.04.2017

ABSTRACT

The effect of sublethal concentrations $^1/_8$ and $^1/_4$ of LC_{50} at 24 h of five pesticides including three organophosphates, Dimethoate, Malathion and Quinalphos, one neonicotinoid Acetamiprid and one biopesticide Neem oil on acetylcholinesterase (AChE) activity in forager bees of *Apis mellifera* was studied. The specific activities of acetylcholinesterase (AChE) were measured in head and thorax of surviving foragers after 24 h of treatment. The data revealed that all the pesticides suppressed the AChE activity but organophosphates had more inhibitory effect on AChE activity as compared to neonicotinoid Acetamiprid. Among organophosphates Dimethoate proved to be most toxic at sublethal concentrations followed by Malathion, Quinalphos and acetamiprid. Significant changes were observed at $^1/_4$ of LC_{50} at 24 hrs but did not have so well defined inhibitory effect at $^1/_8$ of LC_{50} at 24 h Neem oil was found to be nontoxic.

Figures : 02 References : 31 Table : 01
KEY WORDS : Acetamiprid, *Apis mellifera*, Biopesticide, Organophosphates.

Introduction

Pesticides are used on a variety of agricultural crops to control a wide range of pests. A large number of pesticides are being used in crop fields against the different types of pests all over the world. Unfortunately pesticides are not selective to pest species alone. Often non-target insects such as honeybees, which are economically important, are destroyed in the process of pest control[1]. While pest insects are the main targets of manufactured insecticides, non-target organisms such as pollinators may come under their attack affecting about 35 % of the world food crops[30]. The adverse impact that broad-spectrum pesticides have on non-target beneficial insects is widely known to be a major cause of pollinator decline in cultivated areas[7, 8, 10]. No doubt that honey bees are the prominent and economically most important group of pollinators worldwide. Therefore, there is a great concern about the decline of the honey bee population (*Apis mellifera*) in several parts of the world mainly due to improper application of pesticides[29]. Pesticides kill bees during some stage of development (like simple poison) or it affects developmental process in such a way that abnormalities are visible in pupa or adult (amorphogenic action)[17, 18]. Honey bees are of particular interest because they come to contact with various pollutants during their foraging activity, making them a perfect bioassay agent for monitoring heavy metals and pesticide toxicity in urban and rural areas[23,26]. There are several methods to measure the efficiency of pesticides, mainly against their targets which are also applicable for the non-target organisms. Biomarkers have been used to reveal the exposure of organisms to various chemicals in the environment[15].

Enzymes are commonly used as biomarkers or indicators for the spread of environmental pollutants such as pesticides and heavy metals. Acetylcholinesterase (AChE),

ACKNOWLEDGEMENTS : I wish to thank Dr A.K. Saini, Associate Prof. Chemistry and Dr Himshikha Yadav, Asst. Prof. Botany for their valuable help in technical assessment and Mr Ramesh Gangwar for availing and maintaining honeybee samples. I also thank to Principal, Govt. P.G. College, Bisalpur for providing the laboratory facilities.

carboxylesterase and glutathione S-transferase (GST) are examples of these enzymes that can be assayed to indicate any behavioral and functional changes in both target and non-target insects exposed to high or sublethal doses of insecticides. AChE represents a biomarker of neurotoxicity widely used for identifying exposure to chemicals such as organophosphate and carbamate insecticides[4,13,29] and some pyrethroids[3,6]. Other classes of environmental contaminants such as complex mixtures of pollutants, detergents and metals are also involved in AChE reduction[12]. AChE depression has been widely used as a biomarker of general exposure to pollutants, especially organophosphate and carbamate pesticides[28]. However, the activities of other enzymes such as GSTs and carboxylesterase involved in the detoxification and removal of a wide variety of toxic compounds by conjugation or hydrolysis are also monitored[14]. Information on the toxicity of pesticide doses used in the agricultural crops in India or other part of the world on honeybee *A. mellifera* is very important. The present research is to determine the respective toxicity of Organophosphates Dimethoate, Quinalphos and Malathion, one neonicotinoid Acetamiprid and one biopesticide Neem oil on the AChE activity as biomarkers of environmental stress on *A. mellifera.*

Materials and Methods

a) Pesticides

Sublethal formulations of all the five pesticides being used in Indian Agriculture were selected for this study. The sublethal concentrations of all pesticides were determined by acute toxicity assay tests. The detail of the pesticides pursued for the experiments includes Dimethoate [Organophosphate – 30 EC, subtethal conc. 0.0075% ($^1/_8$ of LC_{50} at 24 h) Conc-1, and 0.0150% ($^1/_4$ of LC_{50} at 24 h) Conc-2 supplied by Bayer Ltd, India], Quinalphos [Organophosphate – 25 EC, subtethal conc. 0.01% ($^1/_8$ of LC_{50} at 24 hrs) and 0.020% ($^1/_4$ of LC_{50} at 24 hrs)supplied by Gujrat Pesticides Limited, India] and Malathion (Organophosphate – 50 EC, subtethal conc. 0.0250% ($^1/_8$ of LC_{50} at 24 h) and 0.0500% ($^1/_4$ of LC_{50} at 24 h) Conc-2 supplied by Bayer Ltd, India], one neonicotinoid Acetamiprid-20 SP 0.030% ($^1/_8$ of LC_{50} at 24 h) and 0.06% ($^1/_4$ of LC_{50} at 24 h) supplied by Gujrat Pesticides Limited, India] and *one biopesticide* Neem oil – 25 EC, 0.050% ($^1/_8$ of LC_{50} at 24 h) and 0.10% ($^1/_4$ of LC_{50} at 24 h) supplied by Ozonbiotech Ltd, India.

b) Forager Worker Bees

The experiments were conducted with worker honey bees of *Apis mellifera* (Hymenoptera: Apidae) in Zoology Laboratory of Govt PG College, Bisalpur, Pilibhit. The adult worker bees were obtained from nearby apiary established by Govt Horticulture Department, Bareilly, (UP) India where honey bee colonies were maintained according to the standard commercial methods in apiary. For this kind of risk assessment, forager honey bees are considered the most ecologically relevant when they start performing external tasks[22]. Extensive literature confirms that foragers are those higher than 20 days of age in a typical colony of honey bees[31].Based on farming records, no obvious diseases were observed on units or colonies, and no hives were treated with pesticides. This was confirmed during the collection of bees. Foraging workers were collected[17]. Briefly, four hives were exposed to smoke twice for 30–60 s before collection. Worker honey bees were collected by shaking from the top super or from the front of the hives into a clean and large plastic container. The container was covered with a solid lid, kept in good condition, and transported to the laboratory in 1 hour. The bees were kept in experimental cages (10×7×12 cm) in groups of 50 at 30 ±2 °C with 62 ±5% RH, and fed a 50% (w/v) sucrose solution.

c) Pesticide Treatment

The acute toxicity of the pesticides was evaluated on foraging workers of honey bees (*A. mellifera*) by oral administration through sugar syrup application at controlled laboratory conditions. The worker bees were orally treated with aforesaid pesticides with different concentrations in 50% sugar syrup. Prior to treatment with insecticides, bees were anesthetized by cooling (4 °C for no longer than 3 min) for handling during bioassay techniques. Each treatment of each concentration was composed of three replicates of plastic cups of 25 bees each covered with a nylon mesh with 75 honey bees total/treatment (three replicates with 25 bees/cup). The amount of insecticide solution (20 ml) was applied on a cotton bed and then attached to the upper surface of the nylon mesh cover of each cup (three replicates per each concentration) and bees were left to feed for 24 h by lapping from the fibers of the cotton wool. The control group of Bees was fed with 50% (w/v) sucrose solution. The tests were carried out at 30 ±2 °C with 62 ±5% RH.

TABLE- 1 : AChE activity (ìmol/minute/mg protein) in head and thorax at conc-1 and conc-2 of pesticides in forager bees of *Apis mellifera* using three replicates. Data are expressed as means ± s.d. Mean values followed by same letters are not significantly different (p e" 0.05). Values in parenthesis are % activity of enzyme in treated bees w. r. t. control bees.

GROUPS	TREATMENT	AChE ACTIVITY (mM/min./mg protein)			
		HEAD		THORAX	
		At Conc. -1	At Conc. -2	At Conc. -1	At Conc. -2
CONTROL BEES	Sugar Syrup (50%)	19.33^{a} ± 2.44 (—)	19.33^{a} ± 2.44 (—)	7.72^{a} ± 0.93 (—)	7.72^{a} ±0.93 (—)
TREATED BEES	Neem oil 25EC	18.99^{a} ± 2.39 (98.24)	18.4^{a} ± 3.01 (95.24)	7.55^{a} ± 1.95 (97.80)	7.51^{a} ±1.77 (97.27)
	Acetamiprid 20% SP	18.57^{a} ± 2.17 (96.06)	16.77^{bc} ± 3.17 (86.76)	6.99^{a} ± 1.96 (90.54)	6.43^{b} ±1.23 (83.29)
	Quinalphos 25EC	17.45^{b} ± 3.13 (90.27)	14.39^{e} ±2.42x (90.27)	6.78^{b} ± 2.33 (87.82)	5.89^{c} ±1.17 (76.29)
	Malathion 50EC	16.21^{cd} ± 2.17 (83.85)	14.27^{e} ± 2.96 (73.82)	6.23^{bc} ± 1.01 (80.70)	5.59^{cd} ±0.93 (72.40)
	Dimethoate 30EC	15.72^{de} ± 1.67 (81.32)	13.70^{f} ± 2.36 (70.87)	5.97^{c} ± 0.99x (70.87)	5.21^{d} ±0.97 (67.48)

d) Biochemical Studies

Specific activities of AChE were determined in the head and thorax of the surviving honey bees after 24 h of the oral treatment. Surviving bees were anesthetized by cooling at 4°C for 3 min and the head and thorax were dissected, freed from wings, and rinsed in ice-cooled phosphate buffer (pH 7.0). To measure the activity of the aforementioned enzyme, crude extract of assigned tissues was used. For each crude extract, the particular tissue was collected from the exposed foraging bee workers, weighed, and mixed with the proper volume of extraction mixture to make a 10 % (w/v) extract. The extraction solution consist of 10 mM NaCl, 1 % (w/v) Triton X-100, and 40 mM sodium phosphate buffer (pH 7.4). The tissues were homogenized in the extraction solution using a glass/Teflon homogenizer on ice. The homogenate was filtered through cheesecloth and centrifuged at 10,000 rpm (Rami, India) for 20 min at 4 °C. The tissue pellet was subjected to extraction and centrifugation thrice with phosphate buffer (pH 7.4). The recovered supernatant fraction containing crude enzyme was mixed and used immediately for assaying AChE. All procedures were carried out at 4°C, and all of the experiments were performed in triplicate. Activity of AChE was determined by the colorimetric method[11] using 0.075 M acetylthiocholine iodide (ATChI, Genei-Bangaluru, India) as a substrate. The assay medium (1.5 mL total) consist of 1,420 ìL phosphate buffer (pH 8), 20 ìL of the crude enzyme, 50 ìL of 0.01 M 5, 5-dithio-bis (2-nitrobenzoic acid) (DTNB, Genei-Bangaluru, India), and 10 ìL of 0.075 M ATChI. Over

Fig.1: Showing AChE Activity (mM min./mg protein) in the head of Forager bees of *A. mellifera* exposed to sublethal concentrations of pesticides

10 min incubation at 37 °C, the reaction was monitored using Systronic 166- Spectrophotometer at 412 nm. The specific activity of AChE was expressed as µmoles of ATChI hydrolyzed /min. / mg protein.

e) Statistical Analysis

Statistical analysis was performed using the Microsoft office version 2013 Excel spread sheet in Window 7 and minitab 2010 version 13. Means and standard deviations (s.d.) were determined from three independent replicates of each treatment. The log dose–response curves were used for the determination of LC_{50} values for the insect bioassay according to probit analysis[12]. All data of AChE activities were analyzed by one way analysis of variance (ANOVA) and statistical significance was investigated with Student's t-test at p e" 0.05.

Results

It (Table-1 and Figs. 1 & 2) revealed that the specific activity of AChE in the control bees (19.33 and 7.72 mM/min./mg protein in head and thorax, respectively) was higher than that of the treated bees at both sublethal concentrations. All treatments (excluding neem oil and Acetamiprid at $^1/_8$ of LC_{50} at 24 hrs (conc-1) significantly decreased AChE activity. At $^1/_4$ of LC_{50} at 24 hrs (conc-2) greater inhibition was observed. The most significant inhibitory effect was shown by Dimethoate at conc.-2 where specific activity decreased to 13.70 and 5.21 mM / min. / mg protein in head and thorax, respectively followed by conc-1 of dimethoate decreasing the AChE activity to 15.72 and 6.23 mM / min. / mg protein in head and thorax, respectively. This trend was followed by Malathion, quinalphos and Acetamidprid as shown in table-1. The least but significant decrease in AChE activity was observed in acetamiprid exposed honey bees at Conc.-2 where the specific activity decreased to 16.77 and 6.43 mM / min. / mg protein in head and thorax, respectively. It can be concluded that the AChE activity was strongly inhibited by Dimethoate followed Malathion, Quinalphos, Acetamiprid and then neem oil in descending order.

Discussion

There are a number of insects which are economically very important to human being and environment so the study of the effects of pesticides on insects requires measurement of reasonable and appropriate parameters quantitatively. Unfortunately, there has been little work in insects examining biomarkers for sublethal exposure to pesticides. Honey bees are one of the few types of insects where pesticide-related death incidents are neither intended nor welcomed. Bees may proceed as a reliable biomarker for environmental pollution through their reduced pollination capability, the presence of pesticides residues in their honey, their mortalities, and enzyme inhibition in their tissues when exposed to lethal rates of pesticides[15,19]. Under laboratory conditions, the field recommended doses of pesticides are proven to kill foragers, while sublethal concentrations of pesticides have adversely affect colony function[9,25]. Insecticide actions on the mortality and biochemistry of honey bees have been the subject of many studies[2,8,20,21,24]. The responses of some biochemical parameters, such as alkaline phosphatase, acetylcholinesterase, and glutathione-S-transferase have been extensively characterized in laboratory studies after the exposure of honey bees to various chemicals[3,19]. According to the toxicity response profiles of insecticides on honey bee, *Apis mellifera* they can be considered promising tools for environmental biomonitoring programs. However, no in situ validations have been performed to date. In the present study, the response of AChE was evaluated in vivo to correlate to the sites of inhibition, but also to investigate the possibility to use such enzymes as biomarkers of behavioral and biochemical changes in insects. In the present study, the analysis of data resulting from control and pesticidal administration for 24 h onto foragers allowed excellent comparisons to be made between these toxic pesticides in their formulated form. The results showed differences in the reaction of honey bees to the tested pesticides, probably due to the different modes of action of each insecticide. The present study revealed that all doses of five tested pesticides induced a significant decrease in AChE activity that is maximized at the conc-2. The organophosphates and neonicotinoid acetamiprid are neurotoxic pesticides. These pesticides disrupt the insect's nervous system by prolonged activation of the receptors as they are not hydrolyzed by acetylcholinesterase, leading to death of the insects[5,27,19].

Conclusion

The present study evaluate the toxicity of Dimethoate, Malathion, Quinalphos, acetamiprid and biopesticide Neem oil foraging workers of

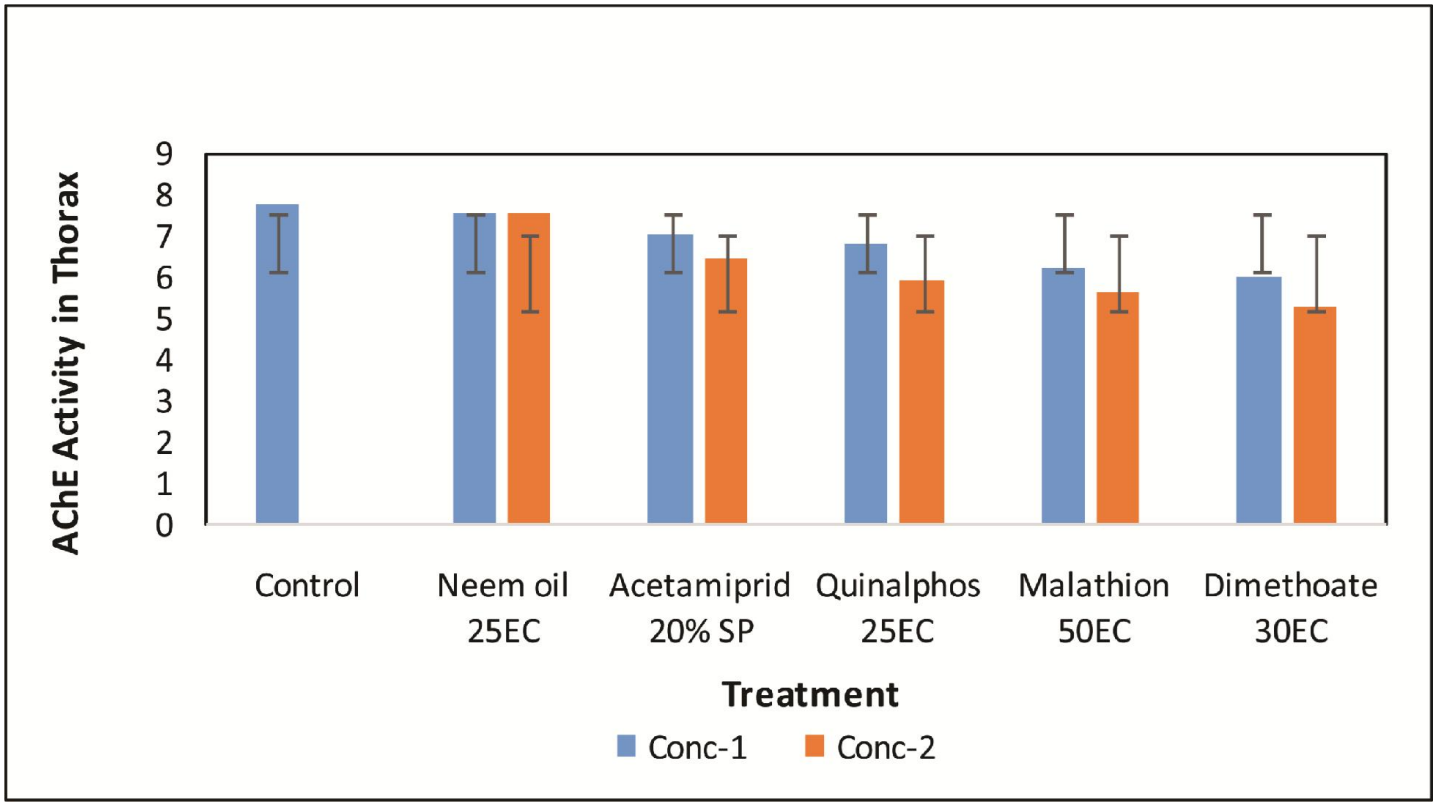

Fig-2: Showing AChE Activity (mM/min./mg protein) in Thorax of Forager bees of *A. mellifera* exposed to sublethal concentrations of pesticides.

honey bees (*A. mellifera* L.) by using two sublethal concentrations of the pesticides for treatment. The activity of AChE enzymes was determined to explore the opportunity of using this enzyme as biomarkers for honey bees exposure to pesticides. The results indicate that Dimethoate was most toxic to bees among the used pesticides especially at conc-2 followed by Malathion, Quinalphos and Acetamipride. It is, therefore, suggested that this insecticide must be used only with greatest care to avoid its drastic negative effects on no target insects essential for agricultural purposes. There were differences in the reactions of honey bees to the other four pesticides, probably due to their different chemical modes of action. This is also to be noticed that acetamiprid was not found as toxic as rest three organophosphates and it also may be due to its totally different chemical structure and mode of action of this pesticide than that of the organophosphates. The biopesticide Neem oil caused no significant inhibition in AChE activity. Thus, our results are of great importance as they can be used as guidelines regarding which pesticides may be toxic to these insects. Our results indicated that AChE activity rapidly decreased after exposure to these pesticides at all tested concentrations, and its specific activity depended on the dose–response curves. Therefore, the Inhibition concentration values can be useful in monitoring the environmental toxicity of these pesticides on honey bees. However, in some cases, the use of some biomarkers as an indicator of sublethal pesticide exposure is not feasible, at least not using easy-to-handle methods in the field.

References

1. ATKINS, E.L., KELLUM, D. AND ATKINS, K.W. (1981) Reducing pesticides hazard to honey bees. Mortality prediction techniques and integrated management strategies. *Division Agric. Sci. University of California, Leaf.* **2883** : 22.
2. ATKINS, E. L., MACDONALD, R.L. AND GREYWOOD, HALE, E.A. (1975) Repellent additives to reduce pesticide hazards to honeybees; Field tests. *J. Environ. Ent.,* **4** : 207-210.
3. BADIOU A., MELED M. AND BELZUNCES L.P. (2008) Honeybee *Apis mellifera* acetylcholinesterase-A biomarker to detect delmathrin exposure. *Ecotoxicol. Environ. Saf.* **69** : 246-253.
4. BANDYOPADHYAY, R. (1982) Inhibition of acetylcholine esterase by permethrin and its reversion by acetylthiocholine, Indian *J. Exp. Biol.* **20** : 488–491.
5. BELZUNCES, L., TCHAMITCHIAN, S. AND BRUNET, J.L. (2012) Neural effects of insecticides in the honey bee. *Apidologie.* **43** : 348–370.
6. BENDAHOU, N., BOUNIAS, M. AND FLECHE, C. (1999) Toxicity of cypermethrin and fenitrothion on the hemolymph carbohydrates, head acetylcholinesterase, and thoracic muscle Na+, K+-ATPase of emerging honeybees (*Apis mellifera mellifera.* L). *Ecotoxicol. Environ. Saf.* **44** : 139–146.
7. BLACQUIERE, T., SMAGGHE, G., GESTEL, C.A.M.V. AND MOMMAERTS, V. (2012) Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology* **21** : 973–992.
8. COSTA, E.M., ARAUJO, E.L., MAIA, A.V.P., SILVA, F.E.L., BEZERRA, C.E.S. AND SILVA, G.J. (2014) Toxicity of insecticides used in the Brazilianmelon crop to the honey bee *Apis mellifera* under laboratory conditions. *Apidologie* **45** : 34–44.
9. DECOURTYE, A., DEVILLERS, J., GENECQUE, E., LE MENACH, K., BUDZINSKI, H., CLUZEAU, S. AND PHAM-DELÈGUE, M.H. (2005) Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honeybee, *Apis mellifera* **.** *Arch. Environ. Contam. Toxicol.* **48** : 242–250.
10. DESNEUX, N., DECOURTYE, A. AND DELPUECH, J.M. (2007) The sublethal effects of pesticides on beneficial arthropods. *Ann. Rev. Entomol.* **52** : 81–106.
11. ELLMAN, G.L., COURTNEY, D., ANDRES, V. AND FEATHERSTONE, R.M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharm.* **7** : 88–95.
12. FINNEY, D.J. (1971) Probit Analysis, 3rd ed. Cambridge University Press, Cambridge, pp. 318.
13. FRASCO, M.F., FOURNIER, D., CARVALHO, F. AND GUILHERMINO, L. (2005) Dometals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. *Biomarkers* **10** : 360–375.

14. GUILHERMINO, L., BARROS, P., SILVA, M.C. AND SOARES, A., (1998) Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned? *Biomarkers* **3** : 157–163.

15. HINTON, B.T., PALLADINO, M.A., RUDOLPH, D. AND LABUS, J.C. (1995) The epididymis as protector of maturing spermatozoa. *Reprod. Fertil. Dev.* **7** : 731–745.

16. HYNE, R.V. AND MAHER, W.A. (2003) Invertebrate biomarkers: links to toxicosis that predict population decline. *Ecotoxicol. Environ. Saf.* **54** : 366-374.

17. IWASA, T., MOTOYAMA, N., AMBROSE, J.T. AND MICHAEL, R. (2004) Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Prot.* **23** : 371–378.

18. JAMES, R. HAGLER, GORDON, D., WALLER AND BRAD, E. LEWIS (1989) Mortality of honeybees exposed to permethrin and combination of permethrin with piperonyl butoxide. *J. Apic. Res.,* **28** (4): 208-211.

19. JOHNSON, R.M., ELLIS, M.D., MULLIN, C.A. AND FRAZIER, M. (2010) Pesticides and honey bee toxicity – USA. *Apidologie* **41** : 312–331.

20. KUMAR, S. AND GUPTA, A.K. (2007) Age dependent study of the impact of certain field pesticides on Acetylcholinesterase activity of *Apis mellifera L. J. Ento. Res.* **31** (2) 115-119.

21. MAYER, D.F., KOVACS, G., BRETT, B.L. AND BRISABRI, B.L. (2001) The effects of spinosad insecticide to adults of *Apis mellifera*, *Megachile rotundata* and *Nomia melanderi* (Hymenoptera : Apidae). *Int. J. Horticul. Sci.* **7** : 93–97.

22. MAYER, D.F. AND LUNDEN, J.D. (1986) Toxicity of fungicides and an acaricide to honey bees (Hymenoptera: Apidae) and their effects on bee foraging behavior and pollen viability on blooming apples and pears. *Environ. Entomol.* **15** : 1047–1049.

23. PICARD-NIZOU, A.L., PHAM-DELEGUE, M.H., KERGUELEN, V.,MARILLEAU, R., OLSEN, L., GRISON, R., TOPPAN, A. AND MASSON, C. (1995) Foraging behavior of honey bees (*Apis mellifera* L.) on Transgenic Oilseed Rape (*Brassica napus* L. Var Oleifera). *Transgenic Res.* **4** : 270–276.

24. PORRINI, C., COLOMBO, V. AND CELLI, G. (1996) The honey bee (*Apis mellifera* L.) as pesticide bioindicator. Evaluation of the degree of pollution by means of environmental hazard indexes. In: Proceedings XX International Congress of Entomology, Firenze, Italy, August 25–31, 444.

25. RABEA, E.I., NASR, H.M. AND BADAWY, M.E.I. (2010) Toxic effect and biochemical study of chlorfluazuron, oxymatrine and spinosad on honey bees *Apis mellifera. Arch. Environ. Cont. Toxicol.* **58** : 722–732.

26. SMIRLE, M.J., WINSTON, M.L. AND WOODWARD, K.L. (1984) Development of a sensitive bioassay for evaluating sublethal pesticide effects on the honey bee (Hymenoptera: Apidae). *J. Econ. Entomol.* **77** : 63–67.

27. SMITH, R.K. AND WILCOX, M.M. (1990) Chemical residues in bees, honey and beeswax, *Am. Bee J.* **130** : 188–192.

28. THANY, S.H. (2010) Neonicotinoid Insecticides. Historical evaluation and resistance mechanisms. *Advances. In: Exp. Med. Biol.* **683** : 75–83.

29. TU, H.T., SILVESTRE, F., SCIPPO, M.L., THOME, J.P., PHUONG, N.T. AND KESTEMONT, P. (2009) Acetylcholinesterase activity as a biomarker of exposure to antibiotics and pesticides in the black tiger shrimp (*Penaeus monodon*). *Ecotoxicol. Environ. Saf.* **72** 1463–1470.

30. VAN, ENGELSDORP, D. AND MEIXNER, M.D. (2010) A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *J. Invertebr. Pathol.* **103** : S80-S95.

31. VELTHUIS, H.H.W. AND VAN DOORN, A. (2006). A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie* **37**: 421–451.