Original Article

HISTOCHEMICAL STAINING OF ACETYLCHOLINESTERASE IN CARNOLIAN HONEYBEE (*APIS MELLIFERA CARNICA*) BRAIN AFTER CHRONIC EXPOSURE TO ORGANOPHOSPHATE DIAZINON

Gordana Glavan*

University of Ljubljana, Biotechnical faculty, Department of Biology, Ljubljana, Slovenia

*corresponding author: gordana.glavan@bf.uni-lj.si
Received: 09 October 2019; accepted 07 January 2020

Abstract

Organophosphate insecticides are known to inhibit the activity of enzyme acetylcholinesterase. They affect olfactory learning and memory formation in honeybees. These insecticides cause mushroom body inactivation in honeybees, but their influence on other brain regions involved in olfactory perception and memory is unknown. The goal of this study was to study the effects of organophosphate insecticide diazinon on carnolian honeybee (*Apis mellifera carnica*) acetylcholinesterase activity in the olfactory brain regions of antennal lobe, mushroom body and lateral procerebrum (lateral horn). The lamina, medulla and lobula of optic lobes were also analyzed. The level of acetylcholinesterase activity was visualized using the histochemical staining method. Densitometric analysis of histochemical signals indicated that diazinon inhibited acetylcholinesterase activity only in the lip of calyces of mushroom body, but not in other analyzed olfactory regions, antennal lobe and lateral procerebrum. The visual brain system optic lobes were also unaffected. This is in accordance with the literature reporting that mushroom body is the main brain center for olfactory learning and memory formation in honeybees.

Keywords: acetylcholinesterase, histochemistry, honeybee brain, mushroom body, olfactory, organophosphate

INTRODUCTION

The survival of managed honeybee populations (*Apis mellifera* L.) is affected by many factors (vanEngelsdorp & Meixner, 2010). In recent years bees have been most negatively impacted by parasites, viral and microbial diseases and especially the parasitic varroa mite (*Varroa destructor)* (Sánchez-Bayo et al., 2016). Pesticides and in particular insecticides are also recognized to be harmful to bees and other pollinators, directly killing them or influencing their overall fitness and cognitive abilities thus impairing the viability of the colonies (Johnson et al., 2010).

One of the classes of commonly used insecticides are organophosphates (Johnson, 2015). Honeybees get into contact with these substances mainly through foraging but also in-hive varroa treatments. Acaricide coumaphos, frequently applied against varroa mites by beekeepers, is an organophosphorus compound (Belzunces, Tchamitchian, & Brunet, 2012). Organophosphates are known to inhibit acetylcholinesterase enzyme (AChE) activity (Johnson, 2015). The most well-known role of AChE in organisms is neuronal. It has a regulatory function at cholinergic synapses, where it rapidly terminates the synaptic transmission through hydrolysis of the neurotransmitter acetylcholine (Thany, Tricoire-Leignel & Lapied, 2010). Studies show that the exposure to organophosphates affects olfactory learning and memory formation in the honeybees (Williamson, Baker, & Wright, 2013; Williamson & Wright, 2013). Consequently, organophosphates could have a great negative impact on a honeybee colony that relies on successful foraging for pollen and nectar through effective navigation and associative learning. Williamson & Wright (2013) and Williamson, Baker, & Wright (2013) utilized olfactory conditioning for the

proboscis extension reflex and discovered that olfactory learning and memory could be affected by organophosphates in different ways. Thus, prolonged exposure to field-realistic concentrations of coumaphos impaired olfactory learning and memory formation in the honeybee, whereas acute exposure to sublethal doses of coumaphos enhanced them. Besides coumaphos, diazinon, another organophosphate pesticide, was shown to inhibit odor learning already at small doses (Weick & Thorn, 2002). In addition, organophosphates also effected locomotion, grooming behavior as well as such symptoms of malaise as abdomen grooming and defecation (Williamson et al., 2013).

The cholinergic neurotransmission system is distributed throughout the insect nervous system and plays a key role in synaptic neurotransmission (Thany, Tricoire-Leignel, & Lapied, 2010). It is also strongly involved in the functioning of the olfactory system, including the mushroom bodies, which are a higher-order insect brain structures mediating multisensory integration, learning and memory (Palmer et al., 2013). The other main olfactory organs and areas are antennae, containing olfactory receptor neurons, antennal lobe and lateral protocerebrum (Galizia & Rössler, 2010). Organophosphate pesticides are known to cause mushroom body inactivation in honeybees (Palmer et al., 2013), but their influence on other brain regions involved in olfactory perception is unknown. Therefore, the goal of this study was to analyze the effect of organophosphate insecticide diazinon on AChE activity in the honeybee brain regions of antennal lobe, mushroom body and lamina, medulla, lobula of optic lobes and lateral procerebrum (lateral horn). The histochemical staining method was used to visualize the level of AChE activity.

MATERIAL AND METHODS

The honeybee subspecies used in the study was the Carnolian honeybee *Apis mellifera carnica*, Pollman 1879 (Insecta, Hymenoptera: Apidae). Honeybee colonies were maintained at the Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, Ljubljana, Slovenia. Healthy and queen-right summer adult worker honeybees were collected from brood combs inside the hive using an aspirator. They were not treated with any chemical substances at least four weeks prior to the experiments.

Chronic feeding exposure to diazinon (5 mg L^{-1}; Diazinon PESTANAL® standard, Sigma-Aldrich Co) was performed as reported by Glavan et al. (2018). Briefly, the honeybees were allocated to four test cages with 15-20 bees per cage and transferred to an incubator in darkness (34 °C, 60% RH; (I-440 CK, Kambič d.o.o., Slovenia)). No food was offered to the bees two hours before the treatment. For the chronic feeding exposure experiment, rectangular wooden cages (10 × 6 × 7 cm; length × width × height) with a mesh were used, described in details by Glavan et al. (2018). All solutions were administered to bees by graduated sterile single-use syringes with cut open ends (polypropylene + polyethylene; Ecoject®, Dispomed, Germany). The feeders were inserted vertically from the top to the bottom of the cages. For the six-day chronic feeding experiment, all four groups of honeybees in each test cage were supplied with two feeders, one containing food and another containing dechlorinated tap water, that were provided ad libitum and renewed every 48 h during the exposure period. The two experimental groups of bees were fed with diazinon (5 mg L^{-1}) in the 1.5 M (51.35% w/v) sucrose solution, while the two control groups only received the 1.5 M sucrose solution. The food was prepared fresh every 48 h from the stock diazinon solution. The stock diazinon solution (20 g L^{-1}) was prepared by dissolving diazinon in absolute ethanol and stored in the dark at 4 °C. The 5 mg L^{-1} concentration of diazinon was selected based on previous results published by Glavan et al. (2018) showing that chronic exposure to 5 mg L^{-1} diazinon concentration inhibits AChE activity in the honey bee brain, while the lower concentrations tested in this study did not have any effect. The chronic exposure experiment was stopped on the sixth day, when a significant dying of honeybees in the two experimental groups was observed compared to control.

For brain cryo-sections preparation, thirteen bees from the control groups and eight bees from the diazinone-treated groups were randomly taken from all experimental cages. The honeybees were first decapitated, and the head capsulae were carefully perforated with high precision micro dissecting scissors in order to facilitate the exposure of tissue to the fixative. The perforated capsulae were dipped in cold 4% phosphate-buffered paraformaldehyde and stored overnight at 4 °C. After fixation, they were washed three times in phosphate-buffered saline. Brains were isolated, washed in phosphate-buffered saline and stored in 30% phosphate-buffered sucrose overnight at 4 °C. The tissue was blotted dry and quickly frozen in tissue-freezing media (Leica) for sectioning. Serial horizontal sections (20 µm) were cut in a cryostat and mounted on poly-L-lysine-coated slides (Sigma-Aldrich Co).

The AChE activity staining was done according to the "direct-coloring" histochemical method introduced by Karnovsky & Roots (1964), which is the modification of the widely used Koelle & Friedenwald histochemical method, in which acetylthiocholine iodide is used as substrate for the detection of cholinesterases. The tissue cholinesterase activity results in the production of thiocholine from acetylthiocholine iodide. Thiocholine reduces ferricyanide to ferrocyanide, which precipitates as copper ferrocyanide resulting in the dark coloration of the tissue at the site of enzymatic activity. In this study instead of acetylthiocholine, iodide acetylthiocholine chloride (Sigma-Aldrich Co) was used as the substrate for AChE. The AChE activity staining procedure started with sections being dipped in 0.1 M sodium acetate buffer for fifteen minutes and then incubated in incubation medium for twenty-five minutes. Incubation media was constituted from 25 ml 0.1 M sodium citrate, 50 ml 30 mM copper sulphate, 325 ml 0.1M sodium acetate buffer (pH = 6), 20 ml 5mM potassium ferrocyanide, 250 mg acetylthiocholine chloride and 80 ml MQ water. After incubation, the sections were rinsed (5 × 3min) in 0.1 M sodium acetate buffer then reacted in a freshly prepared solution of 4% ammonium sulfide for one minute. The staining reaction was terminated with rinses of 0.1 M sodium nitrate buffer (5 × 3 min). They were transferred into the 0.1% solution of silver nitrate for thirty seconds and then rinsed with 0.1 M sodium nitrate buffer (5 × 3 min). Finally, the sections were rinsed with 0.1 M sodium acetate buffer (5 × 3 min) and subsequently stored in a phosphate buffer (PBS) pH 7.4 (137mM Sodium Chloride, 10mM phosphate, 2.7mM Potassium Chloride). The sections were then dehydrated through a graded ethanol series (70, 90, 100%; 4 min in each ethanol solution) followed by xylene (4 min) and coverslipped with Pertex media (Sigma-Aldrich Co.). The brain sections of diazinon-treated and control honeybees were stained simultaneously to ensure the same staining conditions for optimal analysis.

The cover-slipped sections stained for AChE activity were examined and imaged under a Zeiss microscope (ZEISS Axioscope) with an attached digital camera (Leica DFC 290-HD) using the same system settings for all samples. The grey value measurements in the regions of interest and the subtraction of the background signals were performed according to the principles of computerized densitometric image analysis. The regions of interest were antennal lobe (AL), lip of calyces in mushroom body (MB-lip), lamina of optic lobes (OL-LA), medulla of optic lobes (OL-ME), lobula of optic lobes (OL-LO) and lateral protocerebrum (LP). Through the use of ImageJ Software, these brain regions were manually outlined on micrographs in accordance to Calabria et al. (2010), Rybak (2012) and Ito et al. (2014) and afterwards mean grey values were measured. The measured grey values were first calculated for individual bees, through the averaging grey values pertaining to regions of interest of both hemispheres of three brain slices per each honeybee. Subsequently optical density (OD) values were calculated using the equation:

$$OD = log10(\frac{256}{gray\ value})$$

The mean OD values for each analyzed brain region and for the control and diazinon-treated groups were then calculated for statistical analysis. Un-paired Student's t-test was performed on each brain region of interest to evaluate the effects of diazinon on AChE activity by comparing OD brain measurements of control vs diazinon-treated groups. All data are expressed as means ± SD. of OD values. Statistical significance was set at $p < 0.05$.

RESULTS

In the brains of the control untreated bees, a strong AChE signal was observed in antennal lobe glomeruli (Fig. 1A), calyces and β-lobe of mushroom body (β) (Fig. 1C), lamina (la) and lobula (lo) of optic lobes and lateral protocerebrum (lp) (Fig. 1E). The most intense signal compared to all other regions analyzed for AChE staining was observed in the lip of calyces of mushroom body (MB-lip) (Fig. 1C). Relatively weak AChE staining was found in the central part of antennal lobe devoid of glomeruli (Fig. 1A), the lamina of optic lobes (la) (Fig. 1E) and α-lobe and somata (s) of Kenyon cells of mushroom body (Fig. 1C).

Semi-quantitative analysis of AChE histochemical staining, presented in Fig. 1 and Fig. 2., revealed that diazinon statistically significantly lowered the AChE signal in the lip of calyces in the mushroom body (MB-lip) as compared to the control (Fig. 1. C, D; Fig. 2.; unpaired t-test, $p<0.05$). In the other analyzed brain regions of the antennal lobe (AL), lamina of optic lobes (OL-LA), medulla of optic lobes (OL-ME), lobula of optic lobes (OL-LO) and lateral protocerebrum (LP) no statistically significant change in OD values was shown after diazinon treatment as compared to the control (Fig. 1., A, B, E, F and Fig. 2.; unpaired t-test, $p<0.05$). However, in the medulla and lobula of optic lobes diazinon treatment slightly increased the signal intensity as compared to the control (Fig. 2., OL-ME, OL-LO), but this was not statistically significant (unpaired t-test, $p<0.05$).

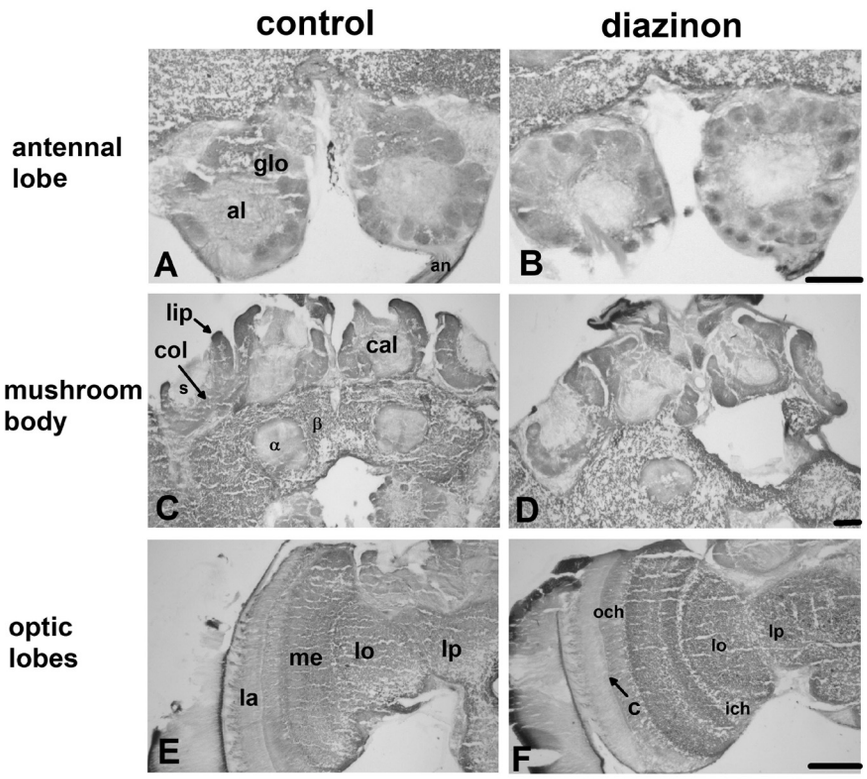

Fig. 1. AChE histochemistry in honeybee antennal lobes (A, B), mushroom body (C, D) and optic lobes (E, F) after diazinon treatment with compared to the control. al, antennal lobe; glo, glomeruli of antennal lobus; an; antennal nerve; cal, calyces; lip, lip of calyces; la, lamina; me, medulla; lo, lobula; s, somata; och, outer chiazma; ich, inner chiazma; c, c-layer; α, α-lobe of mushroom body; β, β-lobe of mushroom body; lateral protocerebrum (lp). Scales 100 μm (antennal lobus: A, B, and mushroom body: C, D) and 20μm (optic lobes: E, F).

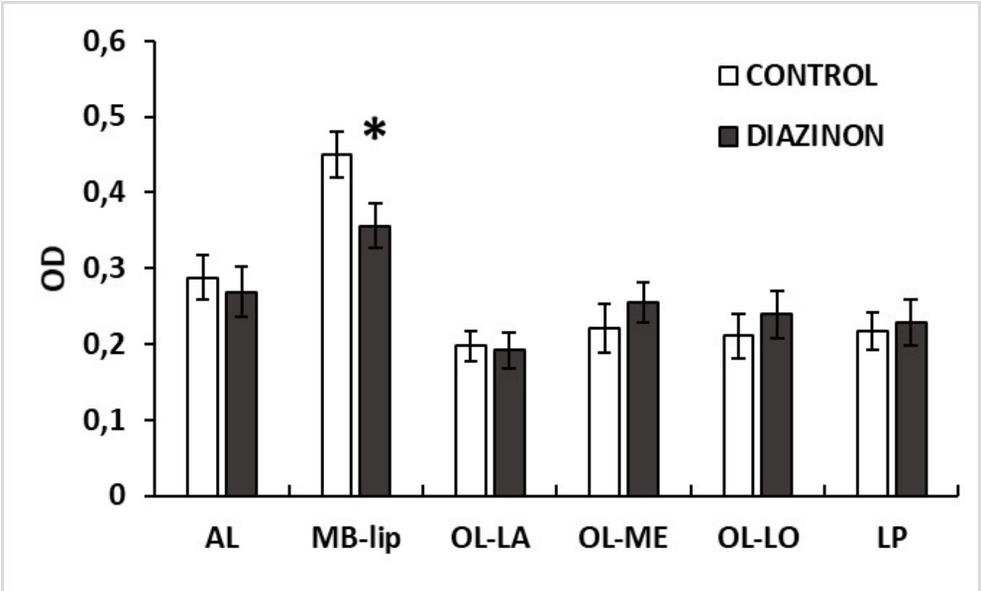

Fig. 2. The effects of diazinon on AChE activity in different regions of honeybee brain. AL, antennal lobe; MB-lip, lip of calyces in mushroom body, OL-LA, lamina of optic lobes; OL-ME, medulla of optic lobes; OL-LO, lobula of optic lobes; LP-lateral protocerebrum. Data are presented as optical density (OD); mean ± standard deviation (SD). Number of bees: N=13 for control group and N=8 for diazinon-treated group;
*significantly different from the control (unpaired t-test, $p<0.05$).

DISCUSSION

The effects of organophosphates on specific honeybee brain regions were studied only in mushroom bodies, using whole-cell recording from Kenyon cells of isolated brains (Palmer et al., 2013). The histochemical AChE staining method was never used for such purpose. Therefore, the aim of this study was to analyze how the organophosphate insecticide diazinon using the histochemical staining method affected AChE activity in different honeybee brain regions, especially those that are a part of the olfactory system and thus to explore which region is most affected by AChE inhibition. In this study histochemical AChE staining showed that diazinon inhibited AChE activity in the lip of calyces in the mushroom body, but not in such analyzed regions as antennal lobe, lateral procerebrum or lamina, medulla, lobula of optic lobes. The goal was also to check the suitability and sensitivity of this method for this kind of research.

Two forms of AChE were found in honeybees. A membrane form that has the most catalytic activity is responsible for synaptic transmission, and a soluble form with relatively unknown function responsible for the small proportion of whole AChE activity (Belzunces, Toutant, & Bounias, 1988). Thus, two separate gene locuses, ace1 and ace2, exist in *A. mellifera* for soluble- and membrane- AChE, respectively (Kim & Lee, 2013). It was shown that in the honeybee head, both forms are expressed in nervous tissue, a membrane AChE form predominantly in the ganglia and head containing the central nervous system (CNS), while soluble is expressed abundantly also in the peripheral nervous system and non-neuronal tissues (Kim et al., 2012). Therefore it can be expected that with histochemical staining both AChE forms are visualized in the brain. However, the staining of a honeybee membrane AChE is probably prevalent as it was shown to have an approximately 2500-fold higher catalytic efficiency towards acetylthiocholine than the soluble AChE (Kim et al., 2012).

Previously, the histochemical AChE staining of honeybee brain has been performed only by Bicker (1999) and Kreissl & Bicker (1989). In both publications, they had performed the AChE staining and acetylcholine receptor immunoreactivity to visualize the cholinergic projections in the bee brain (Kreissl & Bicker, 1989; Bicker 1999). Similar to the results in the present manuscript, a relatively strong AChE signal was

evidenced in antennal lobe glomeruli, calyces and β-lobe of the mushroom body, lamina and lobula of optic lobes and lateral procerebrum (Kreissl & Bicker, 1989). A very intense AChE signal was observed in the lip of calyces of mushroom body as shown also by Kreissl & Bicker (1989) and Bicker (1999). Consistently with the above cited literature, I demonstrated weak AChE staining in the central part of antennal lobe and in the lamina and outer and inner chiazma of optic lobes, α-lobes and somata of Kenyon cells of the mushroom body. However, Kreissl & Bicker (1989) had obtained no AChE signal in the β-lobe of the mushroom body, but in this study there is a strong staining in the β-lobe of the mushroom body visible in Fig. 1. In addition, Kreissl & Bicker (1989) visualized two bands of intense AChE activity in lobulae of optic lobes that was not evidenced in the present study. One explanation for such staining controversy are differences in the protocols for AChE histocehmistry. For instance, Kreissl & Bicker (1989) probably used acetylthiocholine iodide as a substrate for the detection of AChE activity, but I used acetylthiocholine chloride. In general, section thickness, substrate concentration and reaction time could influence the staining intensity and produce an unspecific background signal (Ma et al., 2001).

The histochemical AChE staining showed that diazinon inhibited AChE activity only in the lip of calyces in the mushroom body but not in other analyzed regions. One of the functions of the mushroom body is olfactory learning and memory (Palmer et al., 2013), which could be inhibited by the organophosphate pesticide diazinon (Weick & Thorn, 2002). The main olfactory organs and areas in honeybees are the antenna, antennal lobe, lateral protocerebrum and mushroom body (Galizia & Rössler, 2010). The steps for neural processing of olfactory information are receptor neurons, primary computation in the antennal lobe, and then further processing in such higher-order brain centres as the mushroom body or lateral protocerebrum. Odor information is carried from the antenna to the antennal lobe, where receptor fibres are sorted in numerous glomeruli according to chemospecifities, e.g. primary odour qualities (Heisenberg, 2003). This primary odour information is sent to the lateral protocerebrum and calyx of the mushroom body. These connections are assumed to be cholinergic (Yasuyama, Meinertzhagen, & Schürmann, 2003). Lateral protocerebrum is believed to be involved in the processing of the fine temporal structure of the olfactory input, and important for acute immediate behaviors, but not for memory formation (Heisenberg, 2003).

On contrary, in calyces of mushroom bodies, a multisensorial integration is operated (Dupuis et al., 2012). From my results, I assume that diazinon inhibits the AChE in cholinergic synapses in calyces of mushroom bodies of neuros projecting from the antennal lobe and this is a major site of olfactory learning and memory perturbation induced by diazinon in honeybee. However, according to Ma et al. (2001) and others, conventional histochemistry yields quantitative results with great difficulty from tissue sections and it is more suitable for morphological analysis. Therefore it is very likely that with this method only profound differences in the staining intensity could be observed. After diazinon treatment in this study this was proved for the calyx of mushroom bodies. In other brain regions containing cholinergic neurons the inhibition was expected but not measured, and it could then be speculated that the sensitivity of this histochemical method for visualizing AChE activity is rather low.

In conclusion, the current study reveals that diazinon, which is known to impair olfactory learning and memory, inhibited AChE activity in the honeybee olfactory brain system, but only in the lip of calyces of the mushroom body. Other analyzed olfactory regions, antennal lobe and lateral procerebrum, and optic lobes, comprising the visual brain system, were not affected. These results are more or less expected, as that mushroom body is known to be the center for associative learning and has been implicated in a wide range of behaviors, including olfactory learning. Such advanced histochemical methods as digital scanning densitometry (Ma et al., 2001) should be applied for further quantitative analysis and understanding of the mechanisms of the effects of organophosphates on

honeybee brains, especially systems involved in olfactory learning and cognition.

ACKNOWLEDGEMENTS

Research presented in this paper was supported by the Slovenian Research Agency, through the Integrative Zoology and Speleobiology P1-0184 research programs. The author thanks Kaja Vukotić for technical assistance and Anita Jemec Kokalj for providing the diazinon.

REFERENCES

Belzunces, L.P., Tchamitchian, S., & Brunet, J.L. (2012). Neural effects of insecticides in the honey bee. *Apidologie, 43*(3), 348-370. http://doi.org/10.1007/s13592-012-0134-0

Belzunces, L.P., Toutant, J.P., & Bounias, M. (1988). Acetylcholinesterase from *Apis mellifera* head. Evidence for amphiphilic and hydrophilic forms characterized by Triton X- 114 phase separation. *Biochemical Journal, 255*(2), 463-470. http://doi.org/10.1042/bj2550463

Bicker G. (1999). Histochemistry of classical neurotransmitters in antennal lobes and mushroom bodies of the honeybee. *Microscopy Research and Technique, 45*(3), 174-183. https://doi.org/10.1002/(SICI)1097-0029(19990501)45:3<174::AID-JEMT5>3.0.CO;2-U

Calábria, L.K., Teixeira, R.R., Moraes, V.R., Santos, A.A., Espindola, F.S. (2010). A metallic impregnation technique adapted to study the honeybee *Apis mellifera* L. brain. *Neotropical Entomology, 9*(5), 720-724. http://dx.doi.org/10.1590/S1519-566X2010000500008

Dupuis, J., Louis, T., Gauthier, M., Raymond, V. (2012). Insights from honeybee (*Apis mellifera*) and fly (*Drosophila melanogaster*) nicotinic acetylcholine receptors: from genes to behavioral functions. *Neuroscience Biobehavioral Reviews, 36*(6), 1553-6449. http://dx.doi.org/10.1016/j.neubiorev.2012.04.003

Galizia, C. G., & Rössler, W. (2010). Parallel olfactory systems in insects: anatomy and function. *Annual Review of Entomology, 55*, 399-420. https://doi.org/10.1146/annurev-ento-112408-085442

Glavan, G., Kos, M., Božič, J., Drobne, D., Sabotič, J., Kokalj, A.J. (2018). Different response of acetylcholinesterases in salt- and detergent-soluble fractions of honeybee haemolymph, head and thorax after exposure to diazinon. *Comparative Biochemistry & Physiology Part C: Toxicology and Pharmacology, 205*, 8-14. https://doi.org/10.1016/j.cbpc.2017.12.004

Heisenberg, M. (2003). Mushroom body memoir: From maps to models. *Nature Reviews Neuroscience, 4*, 266-275. http://dx.doi.org/10.1038/nrn1074

Johnson, R.M. (2015). Honey bee toxicology. *Annual Review of Entomology, 60,* 415-434. https://doi.org/10.1146/annurev-ento-011613-162005

Johnson, R.M., Ellis, M.D., Mullin, C.A., Frazier, M. (2010). Pesticides and honey bee toxicity – U.S.A. *Apidologie, 41*(3), 312-331. https://doi.org/10.1051/apido/2010018

Ito, K., Shinomiya, K., Ito, M., Armstrong, J.D., Boyan, G., Hartenstein, V., ... Vosshall, L.B. (2014). A systematic nomenclature for the insect brain. *Neuron, 81*, 755-765. https://doi.org/10.1016/j.neuron.2013.12.017

Karnovsky, M.J., & Roots, L. (1964). A "direct-coloring" thiocholine method for cholinesterases. *Journal of Histochemistry & Cytochemistry, 12*(3), 219-221. https://doi.org/10.1177/12.3.219

Kim, Y.H., Cha, D.J., Jung, J.W., Kwon, H.W., Lee, S.H. (2012). Molecular and kinetic properties of two acetylcholinesterases from the Western honey bee, *Apis mellifera*. *PLoS ONE, 7*, e48838. https://doi.org/10.1371/journal.pone.0048838

Kim, Y.H., & Lee, S.H. (2013). Which acetylcholinesterase functions as the main catalytic enzyme in the Class Insecta? *Insect Biochemistry and Molecular Biology, 43*(1), 47-53. http://dx.doi.org/10.1016/j.ibmb.2012.11.004

Kreissl, S., & Bicker, G. (1989). Histochemistry of acetylcholinesterase and immunocytochemistry of an acetylcholine receptor-like antigen in the brain of the honeybee. *The Journal of Comparative*

Neurology, 286(1), 71-84. https://doi.org/10.1007/BF00318481

Ma, T., Cai, Z., Wellman, S.E., Ho, I.K. (2001). A quantitative histochemistry technique for measuring regional distribution of acetylcholinesterase in the brain using digital scanning densitometry. *Analytical Biochemistry, 296*(1), 18-28. https://doi.org/10.1006/abio.2001.5208

Palmer, M. J., Moffat, C., Saranzewa, N., Harvey, J., Wright, G. A., Connolly, C. N. (2013). Cholinergic pesticides cause mushroom body neuronal inactivation in honeybees. *Nature Communications, 4*, 1634. https://doi.org/10.1038/ncomms2648.

Rybak, J. (2012). The digital honey bee brain atlas. In: *Honeybee neurobiology and behavior–a tribute to Randolf Menzel*. (pp 125–140). The Netherlands: Springer. DOI: 10.1007/978-94-007-2099-2

Sánchez-Bayo, F., Goulson, D., Pennacchio, F., Nazzi, F., Goka, K., Desneux, N. (2016). Are bee diseases linked to pesticides? A brief review. *Environment International, 89-90*, 7-11. http://dx.doi.org/10.1016/j.envint.2016.01.009

Thany, S.H., Tricoire-Leignel, H., & Lapied, B. (2010). Identification of cholinergic synaptic transmission in the insect nervous system. *Advances in Experimental Medicine and Biology, 683*, 1-10. DOI: 10.1007/978-1-4419-6445-8_1

Van Engelsdorp, D., & 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. *Journal of Invertebrate Pathology, 103*(1), 80-95. DOI:10.1016/j.jip.2009.06.011

Weick, J., & Thorn, R.S. (2002). Effects of acute sublethal exposure to coumaphos or diazinon on acquisition and discrimination of odor stimuli in the honey bee (Hymenoptera: Apidae). *Journal of Economic Entomology, 9*(2), 227-236. https://doi.org/10.1603/0022-0493-95.2.227

Williamson, S.M., & Wright, G.A. (2013). Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees. *The Journal of Experimental Biology, 216*, 1799-1807. DOI: 10.1242/jeb.083931

Williamson, S. M., Baker, D. D., & Wright, G. A. (2013). Acute exposure to a sublethal dose of imidacloprid and coumaphos enhances olfactory learning and memory in the honeybee, *Apis mellifera*. *Invertebrate Neuroscience, 13*(1), 63-70. DOI: 10.1007/s10158-012-0144-7.

Williamson, S.M., Moffat, C., Gormesall, M.A.E., Saranzewa, N., Conolly, C.N., Wright, G.A. (2013). Exposure to acetylcholinesterase inhibitors alters the physiology and motor function of honeybees. *Frontiers in Physiology, 4*, 13. http://dx.doi.org/10.3389/fphys.2013.00013

Yasuyama, K., Meinertzhagen, I.A., & Schürmann, F.W. (2003). Synaptic connections of cholinergic antennal lobe relay neurons innervating the lateral horn neuropile in the brain of *Drosophila melanogaster*. *The Journal of Comparative Neurology, 466*(3), 299-315. https://doi.org/10.1002/cne.10867