Journal of Insect Physiology 82 (2015) 85–91

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Effects of environmentally-relevant mixtures of four common organophosphorus insecticides on the honey bee (*Apis mellifera* L.)

Yahya Al Naggar [a,b,*], Steve Wiseman [b], Sun Jianxian [b], G. Christopher Cutler [c], Mourad Aboul-Soud [d], Elsaied Naiem [a], Mohamed Mona [a], Amal Seif [a], John P. Giesy [b,e,f,g,h,i]

[a] Department of Zoology, Faculty of Science, Tanta University, 31527 Tanta, Egypt
[b] Toxicology Centre, University of Saskatchewan, 44 Campus Drive, Saskatoon, SK, Canada
[c] Department of Environmental Sciences, Agricultural Campus, Dalhousie University, Truro, NS, Canada
[d] Biochemistry Department, Faculty of Agriculture, Cairo University, Giza 12316, Egypt
[e] Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, SK, Canada
[f] Department of Zoology, and Center for Integrative Toxicology, Michigan State University, East Lansing, MI, USA
[g] Department of Biology & Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong, Kowloon, Hong Kong Special Administrative Region
[h] School of Biological Sciences, University of Hong Kong, Hong Kong Special Administrative Region
[i] State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing, People's Republic of China

ARTICLE INFO

Article history:
Received 9 March 2015
Received in revised form 15 September 2015
Accepted 16 September 2015
Available online 21 September 2015

Keywords:
Apis mellifera
Organophosphorus insecticides
Proboscis extension reflex (PER)
Acetylcholine esterase
Detoxification genes
Behavior
OPs

ABSTRACT

We assessed whether exposure to environmentally-relevant mixtures of four organophosphorus insecticides (OPs) exerted adverse effects on honey bees. Adult and worker bees were orally exposed for five days under laboratory conditions to mixtures of four insecticides, diazinon, malathion, profenofos and chlorpyrifos at two concentrations. Concentration in the mixtures tested were equivalent to the median and 95th centile concentrations of the OPs in honey, as reported in the literature. Effects on survival, behavior, activity of acetylcholinesterase (AChE), and expression of genes important in detoxification of xenobiotics and immune response were examined. Survival of worker bees was not affected by exposure to median or 95th centile concentrations of the OPs. Activity of AChE was significantly greater in worker bees exposed to the 95th centile concentration mixture of OPs compared to the median concentration mixture. Expression of genes involved in detoxification of xenobiotics was not affected by treatment, but the abundance of transcripts of the antimicrobial peptide hymenoptaecin was significantly greater in worker honey bees exposed to the median concentration mixture. Results suggest that short-term exposure to environmentally relevant concentrations of a mixture of OPs do not adversely affect worker honey bees.

1. Introduction

There is concern that exposure to agricultural pesticides is compromising the vigor of colonies of the honey bee, *Apis mellifera* L. Organophosphorus insecticides (OPs) are one of the most widely used class of pesticides, which in 2008 comprised 22% of world market share of insecticides (Lazonby and Waddington, 2015), and have been implicated in incidents in which bees were poisoned (Fletcher and Barnett, 2003; Kamler et al., 2003). Residues of OPs have frequently been detected in honey bee colony matrices, and their potential hazard to colonies have been previously studied (Al Naggar et al., 2015a,b; Chauzat et al., 2011; Cutler et al., 2014; Mullin et al., 2010; Wiest et al., 2011). While results of some studies suggest that OPs might not directly cause colony failure (Al Naggar et al., 2015a,b), OPs might interact with others stressors or in combination with each other to compromise the fitness of individual bees or colonies (Cornman et al., 2012).

Most studies of toxic effects of pesticides on bees have focused on effects of single compounds. Even though honey bees in the field are rarely exposed to single compounds, few studies have examined toxic effects of mixtures of pesticides on bees (Gill et al., 2012; Johnson et al., 2013; Pilling and Jepson, 1993). Results of surveys in the USA and Canada (Al Naggar et al., 2015b; Mullin et al., 2010), Europe (Chauzat et al., 2011; Wiest et al., 2011) and North Africa (Al Naggar et al., 2015a) have shown that colonies of bees might concurrently be exposed to dozens of different compounds including multiple OPs. In some cases, mixtures of pesticides have been shown to be synergistic, with reported increases

* Corresponding author at: Toxicology Centre, University of Saskatchewan, Saskatoon, SK S7N 5B3, Canada.
E-mail address: Yehia.elnagar@science.tanta.edu.eg (Y. Al Naggar).

http://dx.doi.org/10.1016/j.jinsphys.2015.09.004
0022-1910/

in toxicity as great as 100-fold relative to that which would be predicted from a strictly additive model (Thompson, 1996). For example, exposure to field-relevant concentrations of the neonicotinoid imidacloprid and the OP coumaphos impaired olfactory learning and memory formation in honey bees (Williamson and Wright, 2013). In a study with four common pesticides (fluvalinate, coumaphos, chlorothalonil, and chloropyrifos), exposure to field-relevant concentrations either individually or in mixtures caused significant increases in larval mortality. Synergistic effects were observed with certain binary mixtures, but a combination of the OPs chlorpyrifos and coumaphos resulted in only additive toxicity (Zhu et al., 2014).

The objective of the present study was to assess whether exposure to environmentally relevant concentrations of a mixture of OPs exerts adverse effects on honey bee workers. Effects of the mixture of OPs on survival, behavior, activity of AChE, and expression of genes involved in detoxification and immune response, were quantified. It was hypothesized that exposure of bees to a mixture of OPs that act via the same mode of action could exert adverse additive effects on the endpoints examined in individual bees.

2. Materials and methods

2.1. Pesticides

Diazinon, malathion, profenofos and chlorpyrifos were used in experiments. These four OPs were chosen because they were the most frequently detected OPs in honey bee colony matrices in a recent Egyptian study (Al Naggar et al., 2015a), frequently detected in other studies (Chauzat et al., 2011; Mullin et al., 2010; Rissato et al., 2007; Wiest et al., 2011), and are potentially hazardous to honey bee colonies (Al Naggar et al., 2015a,b; Cutler et al., 2014; Johnson et al., 2010; Mansour, 2004).

Insecticides used in experiments were technical grade (>98% purity, Accu Standard, New Haven, CT). The peer-reviewed literature was examined to determine concentrations of each OP in honey, from which representative median and 95th centile concentrations of each OP was calculated (Table 1). Because honey represents all the nectar sources collected, whether these are treated crops or wild flowers, and represents food consumed by adults and juvenile bees inside the colony, we focused on residues found in honey to derive test concentrations used in our experiments. The median and 95th centile concentrations of each OP were determined. Stock solutions (1000 ppm) of each insecticide were prepared in acetone according to guidelines for efficacy and side effect testing from the European and Mediterranean Plant Protection Organization (EPPO, 1992) and the International Commission for Plant-Bee-Relationships (ICPBR) and then required dilutions were made for the median and 95th centile concentrations of each OP. Insecticides were collectively added to a 500 g L^{-1} sucrose solution. The final concentration of acetone in solutions was 1% (v/v). For all experiments, bees were exposed to: (1) a mixture of the median concentrations of the four OPs; or (2) a mixture of the 95th centile concentrations of the four OPs. A solution of 1% acetone (vol/vol) in 500 g L^{-1} of sucrose was used as a control. Fresh solutions were prepared for each bioassay replicate (i.e. repeat of the experiment).

Table 1
Concentrations of organophosphorus insecticides (OPs) (ng/g, wm) in honey reported in the peer-reviewed literature, nominal (regular font) and dosed (bold font)[a].

OP	Conc. (ng/g, wm)	References	Median[b]	95th centile[b]
Diazinon	14	Wiest et al. (2011)	14.0, **14.7**	33.0, **32.9**
	35	Johnson et al. (2010)		
	0.25	Al Naggar et al. (2015a)		
Malathion	243	Johnson et al. (2010)	122.0, **112.5**	231.0, **217.2**
	0.243	Rissato et al. (2007)		
Profenofos	0.27	Al Naggar et al. (2015a)	0.30, **0.27**	0.30, **0.26**
Chlorpyrifos	3.27	Al Naggar et al. (2015a)	9.0, **7.7**	70.0, **68.8**
	0.015	Rissato et al. (2007)		
	80	Pareja et al. (2011)		
	15	Johnson et al. (2010)		

[a] Dosed concentrations were different from nominal concentrations because it had been measured after treatment sucrose solutions were prepared as a mixture at field relevant median and 95th centile concentrations.
[b] Median and 95th centile concentration were calculated from concentrations detected for each OP in honey in the peer-reviewed literature.

2.2. Exposure protocol and effects on survival

Experiments were conducted in August 2014 with *A. mellifera* workers of indeterminate age obtained from hives maintained in an apiary near the Dalhousie University Agricultural Campus (Truro, NS, Canada). Adults collected from frames without brood were then returned to the laboratory and placed in refrigerator at 4 °C for approximately 10 min to slow movement of bees. Adult workers were then transferred to ventilated transparent round plastic cages (9 cm H × 7 cm diam.), with 20–30 bees per cage. Bees were deprived of food for 4 h prior to commencement of exposures and thereafter fed treatment or control sucrose solution. Access to sucrose solution was through a cotton dental wick that was inserted through a hole in the bottom of the container, which was soaked in sucrose solution via a 50 mm diameter Petri dish placed under the container holding the bees. The Petri dish was covered with a lid and sealed with parafilm to minimize loss through evaporation. Bees in containers were held in an incubator (24 h darkness; 32 °C; ~60% RH) for up to five days and mortality was recorded daily. Each bioassay consisted of three replicate containers per treatment, and the bioassay was done on three separate occasions (i.e. blocks in time). On the fifth day, subsamples of surviving bees were collected from each container to quantify gene expression (Section 2.3) and AChE activity (Section 2.4). Heads for quantification of activity of AChE and alimentary canals for quantification of gene expression were removed from bee specimens by dissection on ice and samples were stored at −80 °C.

2.3. Gene expression

Real-time quantitative PCR (RT-qPCR) was performed to determine the effect of exposure to the mixture of OPs on expression of genes important for detoxification of pesticides and immunity. Total RNA was isolated from alimentary canals of worker bees by use of an RNeasy Lipid Tissue Mini Kit (Qiagen, Toronto, ON, Canada) according to the protocol provided by the manufacturer. Immediately after extraction, the concentration of RNA was determined by use of a Nanodrop ND-1000 spectrometer (Nanodrop Technologies, Wilmington, DE) and first strand cDNA was synthesized from 0.5 μg of RNA by use of a Quantitect cDNA Synthesis Kit (Qiagen). To perform RT-qPCR, samples of cDNA were diluted 1:5 in water that was free of nucleases. Reactions were performed using Quantitect SYBR Green Reagent (Qiagen). Briefly, a separate 50 μL PCR reaction consisting of 2 × SYBR Green master mix, an optimized concentration of gene-specific primers, nuclease free