Original Article

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Acute Oral Toxicity of Neonicotinoid Insecticides to Four Species of Honey Bee, *Apis florea*, *A. cerana*, *A. mellifera*, and *A. dorsata*

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Abstract

Honeybees are important pollinators in agricultural and natural ecosystems. Conservation of wild pollinators as well as managed honeybees are critical for sustainable ecosystem service. Honeybees are exposed to various threats including pesticides. We tested the susceptibility of the neonicotinoids; Thiamethoxam, Imidacloprid, and Clothianidin, and a carbamate pesticide; carbaryl to four species of honey bees, *Apis florea, A. cerana, A. mellifera*, and *A. dorsata* from northern Thailand. Formulated pesticide products were serially diluted and utilized for oral toxicity test. When treated with the recommended concentrations, all tested adult bees were dead within 18 hr for all tested chemicals. Feral honeybee species showed higher LC50 values for 3 neonicotinoids than the managed honeybees. When treated with carbaryl, LC50 values were not different between the honey bee species. The results indicate that tested insecticides would pose risks not only to managed honeybees but also the indigenous honey bee species. Additionally Korean populations of *A. mellifera* showed higher LC50 values for 4 pesticides compared to the Thailand *A. mellifera* population, indicating that there might be complex interactions of differential exposures of those chemicals in agricultural fields and honeybees.

Key words: LC50, Feral honeybee, *Apis florea, A. cerana, A. mellifera, A. dorsata*

INTRODUCTION

Honeybees are known as economically important social insects and provide various products such as honey, royal jelly, propolis, bee pollen and bee venom in a variety of uses. They are also important pollinators in agricultural and natural landscape ecosystems. Honeybees, primarily *Apis mellifera*, remain the most economically important pollinator in crop species (McGregor, 1976; Watanabe, 1994) dependent on insect pollination for their production. In the world, the majority of crops (ca. 71%) are bee-pollinated, accounting for 90% of food supply for 146 countries (Klein *et al*., 2007). Economic contribution of pollination to crop production was estimated to be 2-7 trillion USD worldwide and over 6 billion USD in Korea (Jung, 2008). However, there are increasing concerns regarding pollinator decline including honeybees. Pollinator populations are declining in many parts of the world (Lebuhn *et al*., 2012). Especially, the putative decline of the European honeybee, *A. mellifera* has been reported in developed counties such as USA, Europe, Australia and New Zealand, even though the decline is not a global unidirectional trend (Aizen *et al*., 2009). Because such a large portion of the human food supply is derived from pollinator dependent crops, honeybee decline implies a risk of reduced food production, the so called pollination deficit from pollinator deficit (Kevan, 1999).

Recently habitat deterioration including habitat degra-

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dation and fragmentation of natural landscapes (Thomas *et al*., 2004), higher pathogen prevalence (Colla *et al*., 2006; Cordes *et al*., 2012; Graystock *et al*., 2013; Furst *et al*., 2014), competition between native and invasive species (Goulson, 2003), climate change and agricultural intensification have led to less plant diversity as well as increased usage of chemical inputs such as fertilizer and pesticides (Atkins, 1992; Kluser *et al*., 2007). The consequences of pesticides on pollinators are well documented and understood (Johansen and Mayer, 1990; Sihag, 1995). The non-target effects of neonicotinoid insecticides has been well established and some of these insecticides are already banned for commercial uses in Europe (EU, 2013). In Korea, beekeepers have historically experienced honeybee poisoning by such standard insecticides such as carbaryl (Kim and Jung, 2013b).

A. mellifera has been in the spotlight of attention since the detection of CCD (Colony Collapse Disorder) which resulted in the initiation of multiple conservation efforts. However, information on the Asian species of honeybees relative to the negative impacts of pesticides is limited, even though many Asian honeybee populations are in state of extinction risk such as the indigenous *Apis cerana* population in Korea (Jung and Cho, 2015). In many parts of Asia, *A. florea, A. cerana*, and *A. dorsata* are exploited by humans for honey harvesting as well as for crop pollination (Oldroyd and Wongsiri, 2006). In several Asian countries, European honeybees are well established and function as the primary bee species for honey production (Jung and Cho, 2015) and managed pollination service.

Neonicotinoids are neuro-active insecticides chemically similar to nicotine. Neonicotinoids bind to nicotinic acetylcholine receptors of a cell and triggers a response by that cell. Nicotinic acetylcholine receptors are activated by the neurotransmitter acetylcholine. While low to moderate activation causes nervous stimulation, high levels over stimulate and block the receptors (Yamamoto, 1999) causing paralysis and death. Acetylcholinesterase breaks down acetylcholine to terminate signals from these receptors. However, acetylcholin esterase cannot break down neonicotinoids and their binding is irreversible. Compared to organophosphate and carbamate insecticides, neonicotinoids cause less toxicity in birds and mammals. However, neonicotinoid use has been linked in a range of studies to adverse ecological effects, including honeybee colony collapse disorder (CCD) and loss of birds due to the reduction in insect populations. In 2013, the European Union and a few non-EU countries restricted the use of certain neonicotinioids (Cressey, 2013). Even with the increasing concerns on the toxicity of neonicotinoid pesticides, those are still the most prevailing group of pesticides to control various agricultural arthropod pests. In this study, we compared the toxicity of three neonicotinoid insecticides and one carbamate to four species of honeybees relative to the body size. We also compared the toxicological data of *A. mellifera* from two geographic locations where the use of agricultural inputs are significantly different; Chiang Mai, Thailand and Andong, Korea which could result in evaluating the possibility for using honeybees as bio-indicators to monitor environmental stresses brought by biotic and abiotic factors.

MATERIALS AND METHODS

Test species

Honeybee toxicity tests were conducted at Chiang Mai University (CMU) in northern Thailand in mid January 2015. The tests were carried out on four species of honeybees; *Apis mellifera* L., *A. cerana* F., (domesticated population), *Apis florea* F., and *A. dorsata* F., (feral populations). The feral populations of *A. florea* and *A. dorsata* were collected in situ from the CMU campus. Adult bee workers were placed in refrigeration (4°C) shortly following collection. After cooling, individual bees were transferred into the test system the same day as collection. Experimental populations of *A. cerana* and *A. mellifera* were obtained from the CMU experimental apiary. For *A. florea* and *A. dorsata* mixed ages of workers bees were used in the testing. However, for the managed honey bees, *A. cerana* and *mellifera*, young house-keeping bees were selected for the tests. Body size and body mass were estimated in mm and mg scales, respectively (Fig. 1). *Apis florea* is the smallest and *A. dorsata* is the largest (Oldroyd and Wongsiri, 2006). Differences of body length

Table 1. Insecticides used in this study and their recommended concentration (R.C.)

Chemical class	Common name a.i.*	a.i. (%)	Recommended Conc. (ppm)	Formulation**
Neonicotinoid	Thiamethoxam	10	50	WG
	Imidacloprid	10	50	WP
	Clothianidin	8	40	SP
Carbamate	Carbaryl	50	625	WP

* a.i. = active ingredient.

** WG = Water dispersive granule, WP = Wettable powder, SP = Soluble powder.

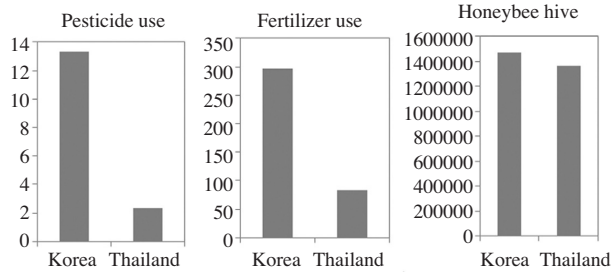

Fig. 1. Environmental indicators, pesticide use (kg/ha, average of 1992-2010) and fertilizer use (Kg/ha, average of 2002-2010) of agro-ecosystems in the Republic of Korea and Thailand (Source: FAOSTAT).

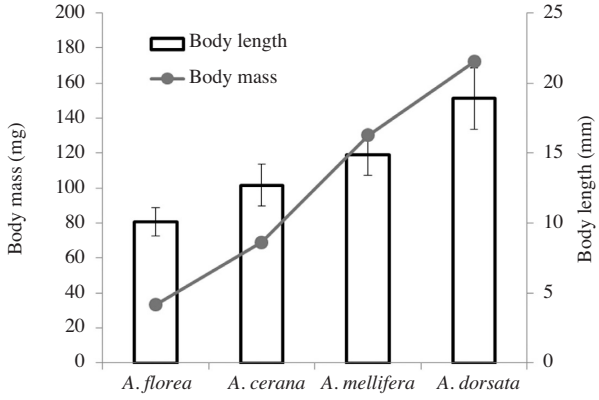

Fig. 2. Body mass (mg) and body length (mm) of the four tested species of honey bee workers.

and mass was *ca*. 2 and 5 times respectively.

Pesticides and test system

Three neonicotinoid insecticides (thiamethoxam, imidacloprid, clothianidin) and one carbamate (carbaryl) were used for the acute oral toxicity tests of four species of honeybees. Commercial formulations available in Korea were used for simulating the actual field situation where formulated insecticides would be applied (Table 1). Individual bees were used in the tests. Individual honey bees were exposed to 1.5ml of 30% sugar solution in which different concentrations of pesticides were added (n=30 or 50). Sugar solution (Pls provide concentration ratio of water and sugar) was provided in a 1.5ml tube inside a 50ml transparent plexiglass cup on which several holes were provided for ventilation. Pesticide concentrations were prepared from recommended concentration to the lower level until 10^{-5} (Table 2). In the control treatment, only sugar solution was provided without any insecticides. After exposure of the individuals to the pesticide contaminated sugar solution, mortality was checked at 1, 4, 8, 12, 24 hours after treatment. If there is no movement of appendages when touched by a hair brush, then the test bee was considered dead.

Additionally, the toxicological data for *A. mellifera* from two geographic locations were compared. For that the same experimental set-up as used in the Thailand study were repeated in Andong, Korea using a Korean population of *A. mellifera* in mid-May, 2015. For the two countries, we collected pesticide and fertilizer use data from the FAO database (FAOSTAT, access at 2015) for the past 20 years, and Korean honeybee hive numbers from FAOSTAT, and for Thailand from Oldroyd and Wongsiri (2006).

Data analysis

Dose-mortality relationships were analyzed based on the treatment concentration rather than treatment dose. In our experiments, it was not possible to measure the amount of oral uptake of the treated pesticide-contaminated sugar solution per each individual bee. Thus, median lethal

concentration (LC50) and median lethal time (LT50) were estimated by probit analysis using Polo Plus program (LeOra software, 2007). T-tests were conducted to compare the differences between the toxicity at the two locations, Andong Korea and Chiang Mai Thailand.

RESULT

Toxicity relative to honeybee species

There were significant differences observed in the LD50 for different pesticides (Pls provide stat values) and also among different honeybee species (Pls provide stat values) (Table 2). However, a consistent size effect was not found among tested chemicals. Thiamethoxam LC50 values at 12 hours after treatment (HAT) were low for *A. cerana* and *A. mellifera* and high for *A. florea* and *A. dorsata*. A similar pattern was found for imidacloprid. Thiamethoxam LC50 values at 12 HAT showed the lowest value for *A. mellifera* and highest for *A. dorsata*. For carbaryl, there was no difference in LC50 values for all species tested. Among the tested chemicals, LC50 values of carbaryl were the highest.

Mortality from thiamethoxam intake occurred earlier than other pesticides (3.2, <1, <1, and 1.5 HAT for *A. florea, A. cerana, A. mellifera*, and *A. dorsata* respectively). Median lethal times at recommended concentrations of each chemical for *A. cerana* were all less than 1 hr. Mortality times lengthened for *A. florea* and *A. dorsata* (Table 3).

Toxicity relative to *A. mellifera* from two locations

When comparing the toxicity data from two locations, the Andong *A. mellifera* population showed much higher LC50 values than Chiang Mai population for all insecticides from 5 times (carbaryl), 40 times (clothianidin) to 100 times (imidacloprid and thiamethoxam) (Table 4). Pesticide and fertilizer use (kg/ha) was 3-6 times higher in Korea as compared to Thailand (Fig. 1).

DISCUSSION

This study clearly demonstrates that the tested pesticides are harmful not only to managed *Apis mellifera* and *A. cerana* but also the feral honeybee species *A. florea* and *A. dorsata*. Even though the distribution of the feral honeybees could be more remote or separated from major agricultural systems, chances for hazardous pesticide exposure exist for feral honeybees (Oldroyd and Wongsiri, 2006). Usually the median lethal dose or median lethal concentration can be used to evaluate the possible pesticide risk to beneficial organisms. Surprisingly LC50 patterns to the tested neonicotinoids were in contrast to our expectation of higher sensitivity for the smaller honey bee, *A.*

Table 2. Median lethal concentration (LC50) of each insecticide to four species of honeybees at 12 HAT.

Species	LC50 (ppm)			
	Thiamethoxam	Imidacloprid	Clothianidin	Carbaryl
A. florea	0.031 a	2.947 a	0.036 b	2.500 a
A. cerana	0.003 b	0.002 b	0.083 b	2.005 a
A. mellifera	0.006 b	0.006 b	0.007 c	5.167 a
A. dorsata	0.088 a	1.923 a	0.220 a	1.118 a

*HAT = Hour after treatment.

Table 3. Median lethal time (LT50) of each insecticide to four species of honeybees at 12 HAT.

Species/ Insecticide	Thiamethoxam	Imidacloprid	Clothianidin	Carbaryl
A. florea	3.2	4.6	2.4	7.5
A. cerena	1<*	1.2	1<*	1<*
A. mellifera	1<*	3.5	1.8	2.5
A. dorsata	1.5	3.9	2.5	3.7

*More than half died within 1 hour.

florea. From the results, managed honey bees, *A. mellifera* and *A. cerana* were more sensitive and resulted in lower LC50 values. Higher LC50 values for *A. florea* and *A. dorsata* were observed. The difference of toxicity patterns found could have behavioral or physiological explanations. Managed species of *A. mellifera* and *A. cerana* could have taken up larger amounts of the contaminated sugar solution even under the disturbed experimental conditions, partly because they would have experienced the sugar solution which beekeepers had provided during dearth periods (Roubik and Buchmann, 1984; Scheiner *et al*., 2003), and partly because these managed honeybees often experience repeated disturbance of their bee hive by beekeepers. On the contrary, feral bees would not take up the pesticide-contaminated sugar solution as readily possibly because of the disturbance or enhanced ability to sense the exotic chemicals. Another possibility comes from the physiological mechanism of detoxification of the pesticide. Du Rand *et al*. (2015) showed that using nicotine alternatives to neonicotinoids, honeybees showed active detoxification of nicotine associated with increased energetic investment and also antioxidant and heat shock responses. The increased energetic investment is significant in view of the interactions of pesticides with diseases such as *Nosema* spp. which cause energetic stress and possible malnutrition.

LC50 values of carbaryl were not different among tested honey bee species. Carbaryl, 1-naphthyl methylcarbamate, is the carbamate insecticide which had been widely used for insect control since 1950s when it was introduced for agricultural pest insect management (Claudianos *et al*., 2006). Thus the bees would have developed resistance to the similar level.

The other finding was that the Korean population of *Apis mellifera* showed much higher LC50 values for the

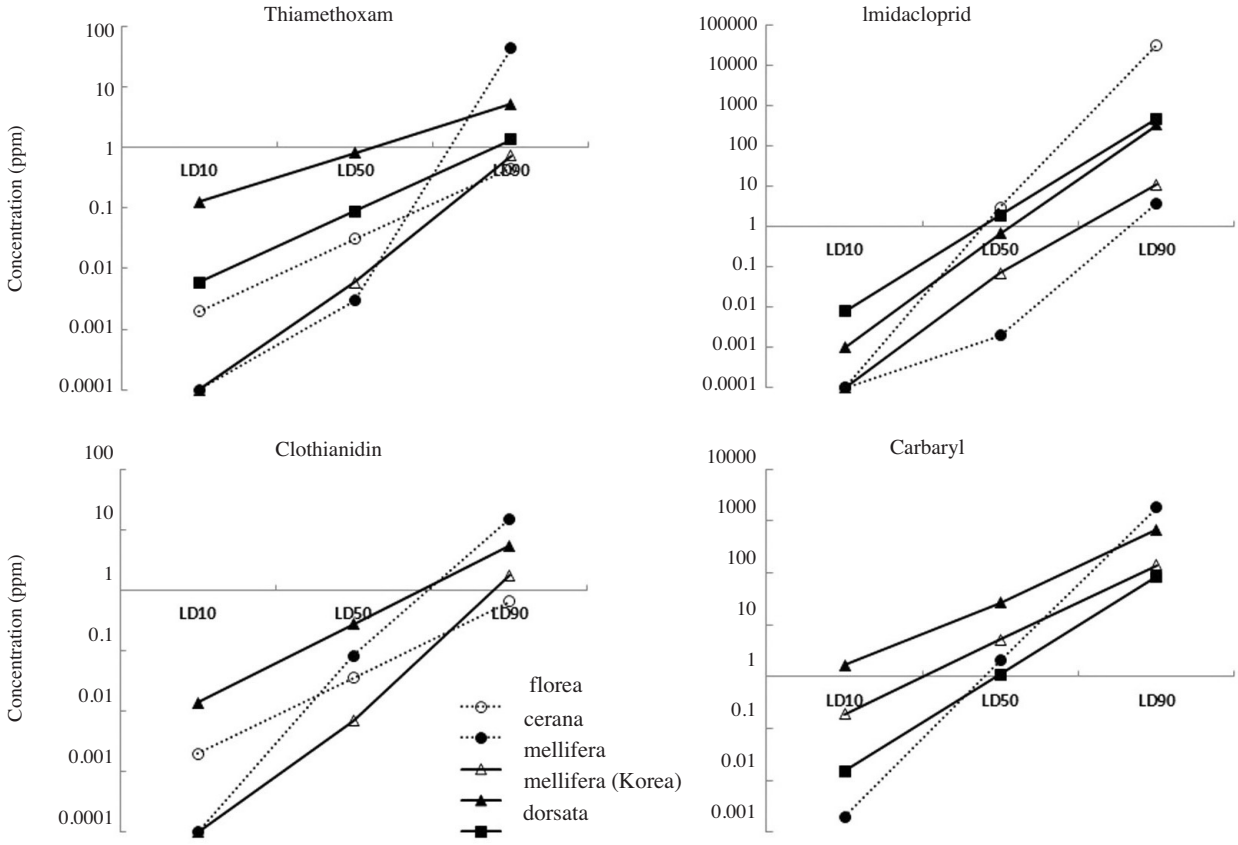

Fig. 3. LC10, 50, and 90 values of 4 insecticides tested for four species of honey bees; *Apis florea, A. cerana, A. mellifera, A. dorsata* from Chiang Mai, Thailand and *A. mellifera* of Andong Korea.

Table 4. Toxicities (LC50 at 12 HAT) of four insecticides to *Apis mellifera* populations at different location in Korea and Thailand

Location & Year	Thiamethoxam	Imidacloprid	Clothianidin	Carbaryl
Chiang Mai 2015	0.006	0.006	0.007	5.167
Andong 2015	0.807	0.669	0.276	26.859

*t-test showed significant difference between the toxicities of two populations.

selected insecticides. The LC50 of carbaryl which is the carbamate insecticide which had been used in agricultural field more than 50 years, showed only a 5 times difference. But the newer neonicotinoid insecticides were about 100 times different. The pattern was similar to the use of pesticide and fertilizer in both countries. However, pesticide and fertilizer use (kg/ha) was 3-6 times higher in Korea than in Thailand. The relationship between the pesticide use and tolerance of non-target beneficial organisms would be required. There are some reports of the different toxicities of insecticides to different honeybees. Colony development and worker honeybee physiological conditions change during the season (Winston, 1987). Even when using *A. mellifera*, substantial differences often emerge when results of toxicity tests on either different genotypes of honeybees or the same honeybee species but different laboratories are compared (Laurino *et al*., 2013). Large differences in the temperature at which the tests are carried out, the age of the honeybees used in the tests, and in the way honeybees are processed and dosed with the toxic substances can lead to substantially different results between different laboratories, even if the same guide lines are followed (Ladas, 1972; Aupinel *et al*., 2009; Medrzycki *et al*., 2012). However in our experiments, every step was standardized and performed by the same persons which partially reduced the chance of errors.

Even though neonicotinoid insecticides are considered less harmful to animals and humans, a range of studies have been done to assess adverse ecological effects, including honey-bee colony collapse disorder (CCD) and losses of birds. In 2013, the European Union and a few non-EU countries restricted the use of certain neonicotinioids (Cressey 2013). Even with the increasing concerns on the toxicity of neonicotinoid pesticides, they are still the most prevalent group of pesticides used to control various agricultural arthropod pests (Cresswell, 2011). Kang and Jung (2010) reported that most honeybee damage is associated with insecticides rather than fungicides or acaricides. Also, even with the same pesticide, toxicities were different to bumblebees and honeybees. A carbaryl residual study on *B. terrestris* and *A. mellifera* showed no toxic effect on bumblebee but highly toxic effects to honeybees (Kim and Jung, 2013a). A recent questionnaire study on honeybee poisoning incidents from beekeepers and apple growers in Korea revealed interesting arguments (Kang and Jung, 2010; Kim and Jung, 2013b). In Korea, RDA (Rural Develop-ment Administration) is the authority for the registration, regulation and safety management of plant protecting products including pesticides, following the pesticide management Act. Careful monitoring of the pesticide use in conjunction with the field risk assessments are required. Also much of the indepth information on the proper use of pesticides during floral blooming was propagated though the agricultural extension and pesticide company marketing teams. Also, reevaluating the hazards they imp-osed (e.g., toxicity information such as LC50, LD50, or hazard quotients (HQ)) could be the primary step, but should expand to the risk assessment in more realistic conditions, which means the environmental monitoring of the pesticides used in agricultural production with emphases on those concerned chemicals.

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