6.5 Monitoring in-hive residues of neonicotinoids in relation to bee health status

John R. Purdy

University of Guelph, Ontario, Canada and Abacus Consulting Services Ltd, P.O. Box 323 Campbellville, Ontario, Canada. E-mail: john@abacuscsl.com , Phone 905 854 2335

Abstract

A field study was done to search for residues of neonicotinoids in twelve honeybee hives in four apiaries in the corn and soybean growing area of southern Ontario, in Canada, and to determine if any bee loss or symptoms of stress were associated with such residues. Dead bees in front of the hive, and live forager bees at the hive entrance and inside the hive were collected. Pollen, honey and nectar were also sampled. Acetamiprid, clothianidin and thiamethoxam and the metabolite TZNG were included in the analysis, and extensive diagnostic tests were done to monitor mites and diseases. Clothianidin, thiamethoxam and TZNG were found in dead bees collected in front of the hives and forager bees from the hive entrance but not in bees from inside the hive. The concentrations found in bees and hive products were below the NOELs for bees, and were not associated with any evidence of stress or bee loss. Mite levels were low, but viruses were frequently found. The pattern of distribution of residues was parallel to what has been reported for other chemicals including chlorpyrifos. Implications of this pattern for the role of the eusocial behaviour of bees in allowing a bee colony to forage on plants bearing natural or xenobiotic toxins are discussed.

Key words honeybee, colony loss, virus, neonicotinoid, resistance

Introduction

Recent reports indicate that neonicotinoids may be harmful to bees under current conditions of commercial use in agriculture, particularly when these compounds are used as seed treatments on maize and soybeans. Many of these reports have been in the form of anecdotal incident descriptions.[1] The attribution of bee losses to pesticides has been the subject of much debate and is not supported by recent extensive reviews of the literature.[2,3,4] _ENREF_3_ENREF_4 Independent statistical records show that the number of bee colonies in both Canada and USA has been increasing since 2006,[4] and annual rates of hive loss are not correlated to agricultural practices.[2] The present work was undertaken to monitor a diverse set of commercial honey bee colonies for neonicotinoids and changes in health and productivity with time in a major corn growing area of Canada. Results from 2013 are presented.

Methods

Study design

The study was set up in 4 apiaries operated by different beekeepers. At each apiary 3 hives were selected arbitrarily for intensive monitoring, but all colony losses were reviewed. Site 2 was on the edge of a maize field (~40 ha), Site 3 was on the edge of a soybean field (~35ha), and sites 1 and 4 were within 500 m of maize fields. An example of the study site layout is shown in Figure 1. The most common cultural practice in the region involves a 3-year rotation of maize, soybeans and wheat/cereal. At each apiary, the colonies were kept in standard Langstroth hives, but site 1 and 4 used solid bottom boards while sites 2 and 3 used screened bottom boards. All beekeepers used 2 brood boxes per hive, and a queen excluder screen was used when honey supers were installed. Sampling and health assessment were done 6 times during the year. The first assessment was at the start of beekeeping activities in May before any crop was planted. Additional assessments were done at planting, post planting, at maize pollination/soy flowering, before winter and in the following spring. A biosecurity protocol followed and care was taken to avoid cross contamination between samples, and to avoid transmission of pests and diseases between hives or apiaries.

Sample collection

All available dead adult bees were collected in front of the hive using a Todd drop zone dead bee trap. The traps were emptied after 2-3 days because the compounds of concern were considered to be unstable in dead bees. When there were significant numbers of dead bees to collect, ten to twenty live forager bees at the hive entrance were collected using a hand held vacuum for comparison of residue levels. At all sampling times, live adult bees from inside the hive were collected by shaking 200-300 bees from a frame of comb obtained from the brood area of the bee colony into a large paper-lined funnel, which directed the bees into a polyethylene sample container. The paper was replaced and the funnel was washed with isopropyl alcohol and dried between samples. Samples (10-20 g) of hive pollen (bee bread), nectar and capped honey were collected into polyethylene sample vials from honeycomb frames where sufficient material was available in the hive using a flat metal blade of a hive tool. Pollen (10-15 g) was also collected from forager bees using a standard Better Bee® commercial pollen harvesting trap. All samples were labelled, sealed and packed in a re-sealable polyethylene bag. The samples were transferred to a portable freezer and kept below -15°C until they were analyzed.

Analysis

The analytical work was done by Activation Laboratories in Ancaster, Ontario, by LC-MS/MS using a method based on the QUECHERS method.[5] Neonicotinoids acetamiprid, clothianidin and thiamethoxam and the metabolite thiazolylnitroguanidine (TZNG) were included in the analysis. The limit of quantitation (LOQ) was 0.3 µg L^{-1}, which was equivalent to approximately 0.03 ng/bee for 100 mg bees. The method was modified to include isotopically labelled internal standards to eliminate matrix effects.[6] The LOQ for pollen, honey and nectar was 0.6 µg L^{-1}. These LOQ values were set well below the No Effect Level (NOEL) for the compounds of interest.[7]

Health assessment

Bee colony health was assessed at each sampling interval. The hives were opened and a frame-by-frame inspection was done to check for visible symptoms of disease or stress, and to determine the population of bees and presence and status of the queen. Samples were collected and sent for assay by at the National Bee Diagnostic Centre (NBDC) Lab in Beaverlodge, Alberta to determine *Varroa* mite population, American and European foulbrood, two species of *Nosema*, and Viruses. RT-PCR methods were used to detect low levels of the foul brood bacteria, to distinguish between *Nosema ceranae* (Fries) and *Nosema apis* (Zander) and to detect 7 viruses (acute and chronic bee paralysis, Isreali acute bee paralysis, black queen cell virus, deformed wing virus, Kashmir bee virus, sacbrood) known to cause colony loss were detected using R-PCR.[8] Tracheal mites (*Acarapis woodi*, Rennie) were absent in the initial set of samples, and have not been found in the study area for many years, so they were not included in any subsequent testing.

Results:

Analytical Results:

None of the test compounds was detected in bees (60 samples) collected from inside the hive. Dead bee samples (12 samples) were obtained during the season from three of the four apiaries. There were too few dead bees in the collection traps (<5 g) for other hives and at other time intervals to provide enough sample to analyse. The results for these samples and the comparison samples of live foragers collected with them are listed in Table 1. Clothianidin was found in 10 of the 12 dead bee samples (83%) and its degradation product TZNG was found in 8 of the 12 samples (67%). Most detections occurred in the samples collected at planting. At one apiary, detections also occurred in the post-plant samples, and three detections of thiamethoxam occurred in live foragers at planting time. Note that clothianidin is formed during degradation of thiamethoxam .[7]

The maximum concentration detected and the frequency of detection in hive pollen, pollen collected from forager bees, nectar and capped honey are listed in Table 2. The mean or median values for pollen, nectar and capped honey were below the LOQ and are not included in the table. The absence of residues at the time when the maize was producing pollen and when the soybeans were flowering indicates that these crops were not preferred forage for bees in the study area.

Colony Health

All honeybee colonies in the study were considered to be healthy by the beekeepers, and in visual inspections done in the field by study personnel. The hive populations increased rapidly before, during and after planting due to good weather and ample food resources. The growth was so rapid that the beekeepers had difficulty preventing loss of colonies due to swarming. The diagnostic results showed that the levels of *Varroa* mites were low. *Nosema*, American foul brood and European foul brood were occasionally found by RT-PCR methods at NBDC, but always below pathological levels. However, all the adult honeybee samples (55) collected throughout the 2013 season contained at least one virus; over 50% had more than three viruses. Sacbrood was most common, but deformed wing, paralysis and black queen cell viruses were also frequently detected in adult worker bees. Impaired and dying bees collected in front of the hives also had virus diseases, and it appears that these bees are evicted from the colony as part of the hygienic behavior of the honeybees, so that the levels of viruses in the colony are kept low enough for the colony to survive and grow. Honey yields (average 40±11 kg/hive) were at or above normal in all of the hives except those affected by swarming, which occurred in mid to late season.

Discussion

All colonies were in rural agricultural areas where the corn-soybean-wheat crop rotation is common. All were close to corn and soybean fields and were considered to be healthy by the beekeepers. The colonies were in apiaries surrounded by corn and soybean fields; one apiary had more than 50 hives placed directly alongside a corn field and another was beside a soybean field. This makes the results representative of a worst-case potential exposure to neonicotinoid residues. The concentration and frequency of detection in the analytical results were similar to those from incident reports in the area.[7] Since adverse effects were rare, there can be no correlation between the presence of neonicotinoid residues found and signs of stress such as slowed development, reduced honey yield or the presence of viruses. When residues of neonicotinoids were found early in the season, the levels found were below the NOEL.[1] Based on the maximum dietary intake of nectar and pollen by honeybees,[9] the amounts found in nectar, honey and pollen (Table 2) were also harmless. This outcome is in line with the findings of most recent literature reviews.[2,3,4]

The absence of residues in the hive bees shows that these bees metabolize the residues they ingest from pollen and nectar quickly enough to prevent transfer of significant amounts of residue to the bees they feed by trophallaxis. For comparison, the residues of chlorpyrifos in nurse bees was found to be 25% of the level in bee bread.[10] The schematic diagram in Figure 2 below shows the physiological separation of the hypopharyngeal and mandibular food glands from the honey stomach and digestive tract of the honeybee worker.

Honeybees have long been known to forage for pollen and nectar on plants such as tobacco or almonds that contain toxic natural compounds, yet they do not appear to have developed increased tolerance for these toxins.[11] Similarly, honeybees have been maintained in agricultural environments where exposure to pesticide residues may occur. Despite widespread exposure to pesticides[12,5] honeybees have not developed tolerance (or "resistance") in the way many other insects have. It has been reported that honeybees have an uncommonly low number of genes for enzymes like cytochrome P450 that are responsible for detoxifying such material.[13] The same authors suggested without proof that the highly eusocial behavior of honeybees evolved to isolate and protect the brood and reproductive castes of bees in the colony from food-borne toxins. Only the oldest and most expendable workers are involved in foraging outside the hive and are directly exposed to environmental stressors. This makes detoxification enzymes unnecessary.

The present work provides support for this hypothesis. Queen bee larvae and adult queens obtain food and water exclusively via a secretion – royal jelly – from the mandibular and hypopharyngeal glands of nurse bees which do not leave the hive. When they do leave the hive they stop being nurse bees. All bee larvae are fed a similar secretion for the first three days after hatching, followed by a mixture of pollen, honey, water and this glandular secretion.[14] Therefore the Queen, the young larvae and to some extent older brood and drones are protected from exposure to toxins in food that is brought into the hive. This enables honeybees to forage on a wider range of plant species, which is an evolutionary advantage.[13] It follows that when honeybees were introduced into new agricultural ecosystems as occurred when they were brought to North America, they could immediately utilize pollen and nectar from plants such as tobacco that contain toxins.

Further support for this hypothesis comes from work with chlorpyrifos fed to bees as residues in almond pollen. There was a reduction in concentration of nearly 1000-fold between the pollen and the royal jelly fed to the queen larvae.[10] In the results listed in Table 1, the pattern of residues is similar. The absence of detectable residues of neonicotinoids in the adult bee samples collected inside the hive at the same time as the samples of forager bees, nectar honey and pollen in which residues were found is evidence that the live bees can digest neonicotinoids fast enough to prevent exposure of the brood or reproductive castes.

Thus the eusocial behavior of honeybees is itself a new mode of pesticide tolerance. It protects the brood and the sexually reproductive castes in the colony, from environmental toxins, natural or manmade. There is no selection pressure that would lead to traditional metabolic forms of increased tolerance to pesticides. Figure 3 illustrates the layers of protection afforded by the colony order from physical chemical and biological stressors. If a food resource is highly toxic to bees, the scout bees that will not return to the hive and no foragers will be recruited to that resource. Very few bees would be lost.[15] At lower levels of toxicity, the scouts might recruit foragers to the resource but they would not be productive and the source would be abandoned. If residues are returned to the hive, they might affect the hive bees that receive them, but as noted above the reproductive castes are protected.

Clearly this defense mechanism can be overwhelmed in extreme cases by pollen borne toxins or pesticide overexposures, and although the relevance to pesticide tolerance was not recognised, some of the older literature also supports this concept[16]. This is analogous to the level of immunity to diseases found in insects that lack an adaptive immune system like that found in mammals, which has been called "innate resistance". It comes from such things as resistance of the insect cuticle to penetration by pathogens. It follows that the form of tolerance to pesticides and other environmental toxins described above can be called "innate tolerance" to distinguish it from acquired tolerance. This innate tolerance to chemical stressors explains why honeybees do not need to develop the metabolic tolerance to pesticides commonly seen in other insects.[17]

In any case, it is essential for risk assessment to define the individual contributions to the overall dose vs time via the various potential routes of exposure and the distribution of the dose among castes, task groups and life stages in the colony. A revised honeybee exposure conceptual model has been proposed separately to describe the potential routes of exposure of bees to pesticides and to incorporate these findings for risk assessment (J. Purdy, published herewith).

The frequency of occurrence of disease organisms must also be considered, in pesticide risk assessment, particularly viruses. Virus diseases are characterized by periods of apparently benign presence, with episodes of exponential virulence, the symptoms of which are identical to those claimed for neonicotinoid incidents[1,18]. Sacbrood virus shows characteristic symptoms in larvae but cannot be visually diagnosed in adult bees. Knowledge of bee viral disease has lagged far behind the understanding of these diseases in medicine and agriculture; there are no established treatment thresholds or treatments for these highly contagious and infectious diseases at the colony level[19]. Quantitative diagnostic methods for practical use by beekeepers are only in the development stage. Most qRT-PCR methods only give the virus titer relative to that of a host RNA.[8]

The eusocial behavior of honeybees imparts a degree of innate tolerance to diseases and parasites. But the defense against disease and parasites differs from chemical stressors in ways that may permit differential diagnosis (Figure 3). Several mechanisms of innate disease tolerance are known. Figure 3 shows how parasites like *Varroa* mites and the viruses they carry go directly to the larvae in addition to attacking the adults. Other viruses including sac brood do not depend on mite vectors but are transmitted sexually or by the fecal-oral pathway or in food sharing. [8] They bypass the defense barriers, and this is the key to the ecological success of these pests and diseases. The colony responds to biological threats by expelling sick bees from the hive, and by attempting to outpace the loss of individuals by increased egg-laying. If these are overwhelmed, the hive may be killed rapidly or undergo a slow decline with classical symptoms of impaired and dying bees in front of the hive and depletion of the adult worker population. Sacbrood infected nurse bees become foragers earlier leading to a shorter life span. Defensive bees may pick the body hairs off diseased individuals leading to "black bees". Bees with paralysis symptoms are also removed from the hive. These bees are refused food and die with proboscis extended. [8,18] They are often among the dead and impaired bees in front of a hive. From the above discussion and Figure 3, it appears that when the queen, drones and or larvae are affected in a declining hive it is an indication that the hive is being affected by disease and not chemical stress. This distinction may aid in diagnosis of health effects.

While many consider viruses to be insignificant, there is no doubt that they cause major outbreaks of disease and colony loss.[20,19] Since viruses disease are present in all life stages but not always visible, and they produce the symptoms that have been attributed to neonicotinoids including hive loss, it is understandable that in the absence of reliable methods, misdiagnosis may occur.[18] Additional work is in progress to extend and confirm the findings presented herein.

Conclusions

The concentrations of neonicotinoids found in honeybees from colonies placed adjacent to or near maize or soybean fields were below the NOEL and were similar in amount and frequency to those found in samples from bee loss incident reports by PMRA, but the bee colonies were found to be healthy and unaffected. Among 55 adult bee samples, all had at least one significant virus and >50% had more than three. The bees appeared to withstand this, but viruses are characterised by episodes of exponential virulence; there is concern that incidents of colony loss may occur and could be incorrectly attributed to any chemical that might be detected. The results support the hypothesis that the eusocial behavior of honeybees makes the colony less susceptible to pesticides and allows them to forage on a wider range of plants including toxic species. Determination of the distribution of residues among castes, task groups and life stages in the colony is essential for risk assessment. Honeybees have innate tolerance of environmental toxins through isolation of the castes and task groups involved in reproduction. Since parasites and disease bypass this mechanism, involvement of larvae and queen may be useful to distinguish chemical from biological effects.

Acknowledgements:

Tables

Table 1 Residues of neonicotinoids in samples of adult honey bees

		Clothianidin (µg L^{-1})			TZNG (µg L^{-1})			Thiamethoxam (µg L^{-1})		
Site No.	Hive No.	Dead Bees	Fora-gers	Live Hive Bees	Dead Bees	Fora-gers	Live Hive Bees	Dead Bees	Fora-gers	Live Hive Bees
At Planting										
	1	1.1	-- [a]	--	0.9	--	--	--	--	--
1	2	--	--	--	--	--	--	--	--	--
	3	1.0	--	--	0.5	--	--	--	--	--
	1	2.4	1.2	--	0.9	--	--	--	--	--
2	2	0.6	0.9	--	0.6	--	--	--	0.3	--
	3	1.0	0.7	--	--	--	--	--	0.8	--
	1	0.8	--	--	0.3	--	--	--	1.1	--
3	2	0.4	--	--	--	--	--	--	--	--
	3	--	--	--	--	--	--	--	--	--
	1			--			--			--
4	2			--			--			--
	3			--			--			--
Post Planting										
	1	1.9	0.4	--	1.1	--	--	--	--	--
2	2	1.3	1.1	--	0.6	--	--	--	--	--
	3	--	0.6	--	--	--	--	--	--	--

a) Samples with no detectable residue (<0.3 µg L^{-1}) are listed as --. No residues were detected at later times during the season. No acetamiprid was detected in the bees.
b) Shaded areas indicate no sample.was collected.

Table 2 Maximum concentration (µg L^{-1}) of neonicotinoids in samples of hive materials (% of samples with detected residue)

Sample Type	Acetamiprid	Clothianidin	TZNG	Thiamethoxam
Honey	8.2 (3.3)	0.0	0.0	1.2 (13.3)
Nectar	2.1 (9.4)	0.0	0.0	1.0(5.7)
Hive Pollen	1.9 (9.4)	8.4 (36.5)	2.9 (5.8)	14.7 (25)
Forager Pollen	5.3 (7.1)	8.4 (19)	2.8 9.5)	3.4 (21.4)
Wax	7.2 (9.6)	0.5 (3.7)	1.7 (7.4)	0.5 (1.9)

Illustrations

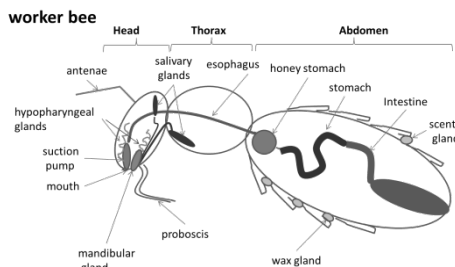**Figure 1** Example of the layout of study sites

Figure 2 Separation of food producing glands from the honey stomach, and digestive tract of the honeybee
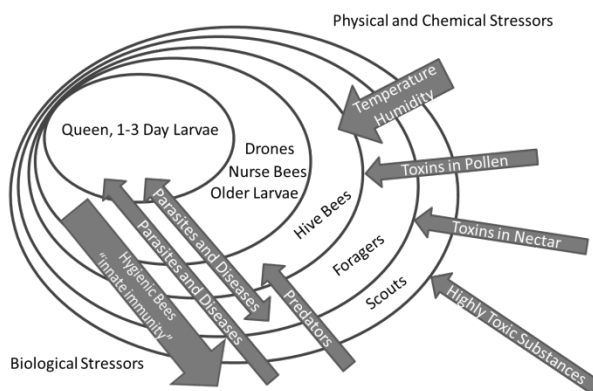

Figure 3: Layers of isolation from external stressors in the social order

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