1.5 Distribution of residues of neonicotinoids in the hive and in bees in relation to bee health

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Abstract

A field study was done to search for residues of neonicotinoids in 15 honeybee hives, in 5 apiaries to determine if any bee loss or symptoms of stress were associated with such residues. The apiaries were adjacent to corn or soybean crop fields in southern Ontario, and Quebec, Canada. Samples of healthy adult bees, larvae, impaired bees with symptoms of intoxication, black bees and dead bees were analysed for acetamiprid, clothianidin, imidacloprid, thiamethoxam, and the metabolite TZNG. Neither the concentrations of the individual compounds found nor the aggregate exposures to multiple compounds were associated with any evidence of stress or bee loss. Extensive diagnostic tests were done to monitor mites and diseases, and hive weights were monitored. Viruses were frequently found in all bee sample types. Over 90% of impaired bees had viruses, but 20% or less had any of the test compounds and only at low levels (<0.05 ng/bee) of neonicotinoids. 77% of black bees had viruses but none of the test compounds was detected in these bees. Method verification, distribution of residues in the colony, assessment of hive scale results, calculation of the combined effects, implications for diagnosis, and risk assessment will be discussed.

Background

A field study was done to search for residues of neonicotinoids in 15 honeybee hives, in 5 apiaries to determine if any bee loss or symptoms of stress were associated with such residues. The apiaries were adjacent to corn or soybean crop fields in southern Ontario, and Quebec, Canada. The design of the study and preliminary results from four of the sites was reported at the Ghent ICPPR meeting in 20141. Figure 1 shows a representative site layout. This report covers the method verification, analytical results, virology, and hive scale results for the completed study. Inclusion of product names in this report does not imply endorsement.

Figure 1: Layout of the hives at one of the study sites

Methods

The analytical method was based on the QuECheRS2 method with LCMSMS detection modified to achieve desired sensitivity and adapted to the various matrices. Acetamiprid (ACM), clothianidin (CLT), imidacloprid (IMI), thiamethoxam (TMX) and the metabolite TZNG were included in the analysis.

Virology was done by measuring median fluorescent intensity in the Quantigene® assay 3. The measure values with background subtracted were normalized using three honeybee genes.

Samples with a low control gene signal were excluded. Values below the Limit of Quantitation (LOQ) were reported as "trace". The bee viruses included were: Acute Bee Paralysis Virus (ABPV), Black Queen Cell Virus (BQCV), Chronic Bee Paralysis Virus (CBPV), Deformed Wing Virus (DWV), Replicating DWV (DWVR), Israeli Acute Paralysis Virus (IAPV), Replicating IAPV (IAPVR), Kashmir Bee Virus (KBV), Sacbrood Virus (SBV) and Varroa Destructor Virus (VDV). As these are RNA viruses, and the RNA is unstable in dead bees, only live and impaired bees could be assayed for viruses.4

Hive weights were recorded 4 times/hr using Beewatch hive scales5 (http://beewatch.de/kontakt).

Samples of honeybees, nectar, capped honey, pollen/bee bread from the brood area, wax, and pollen from foragers that were collected using a front-mounted Better Bee pollen trap (https://www.betterbee.com) were collected from the hives 5 times during the season along with colony condition assessments. The bee samples included larvae, normal brood area bees, and black bees (hairless) from inside the hive and returning foragers at entrance, dead bees outside hive, and "impaired bees" (live but unable to fly, trembling, uncoordinated) outside the hive entrance. The sample sizes were 5-10 g for pollen, nectar, and honey; at least 10 individual late stage larvae, at least 10 individual foragers, black bees or impaired bees, and up to 300 hive bees from the brood area.

Results

Method verification – Samples collected for analysis or assay must be minimal but large enough to be representative and to allow for multiple subsamples (for pesticides, viruses, etc.). Subsamples or aliquots must be representative, randomized, reproducible and large enough to support the desired limit of detection for each measurement to be done. While methods for sample types other than bees are relatively well documented6-8, verification of sample sizes, subsampling, and sample stability were required for the bee samples. Unlike other hive materials honeybee samples contain discrete units with non-uniform concentrations representing a significant proportion of the sample. This makes it difficult to obtain representative subsamples. How many bees make a representative subsample?

The method verification for combined analysis and virology on bee samples was done using bees from one of the samples of live bees known to contain residues on TMX. Replicate random subsamples of 1 and 5 bees were taken from this sample and a sample of 100 bees was taken for comparison. The samples were ground to a uniform slurry in 1.6 mL of water and Duplicate Subsamples of 25 mg samples of slurry were taken for virology. The remainder was analyzed for neonicotinoids. The 100 g sample was frozen in liquid nitrogen and ground to a fine powder. Five replicates of 0.5 g (= 5 bees) were taken for chemical analysis from homogenized powder and analyzed as for the smaller samples. The results are in Tables 1 and 2.

Table 1 Variability vs sample size for honeybee samples- Virology

Sample Description	Statistic	BQCV	DWV	IAPV
single bee	**Mean**	27.2	24.84	31.49
	ST Dev	1.73	5.41	1.56
	CV (%)	**6.35**	**24.77**	**4.96**
5 bees	**Mean**	26.18	26.71	32.6
	ST Dev	3.74	2.94	3.89
	CV (%)	**14.29**	**11.01**	**11.95**
100 bees	**Mean**	23.77	18.35	33.63
	ST Dev	1.56	2.39	0.77
	CV (%)	**6.58**	**13.02**	**2.28**

Results from 5 replicates

Table 2 Variability vs sample size for honeybee samples - Neonicotinoids

Sample Description	Replicate	weight (g)	TMX (PPB)	Mean	ST Dev	CV (%)
single bee	1	0.112	0.0	7.8	17.45	224
	2	0.074	0.0			
	3	0.113	0.0			
	4	0.082	39.0			
	5	0.112	0.0			
5 bees	1	0.36	4.59	1.1	1.96	178
	2	0.474	0.46			
	3	0.429	0.20			
	4	0.465	0.0			
	5	0.483	0.26			
100 bees	1	0.519	1.17	1.06	0.24	23
	2	0.524	0.96			
	3	0.504	1.31			
	4	0.516	1.17			
	5	0.501	0.69			

These results show that variability is much lower for virus detection than for chemical analysis. The results from the subsamples from 100 bees reflect the variability of the method, and the results from single bees are similar because nearly all bees have similar levels of the virus. The CV of the analytical results for subsamples of 100 bees was not much higher than for virology Table 1 and 2, but the CV's for 5 bees and single bee are much higher (Table 2). The individual bee results show that the proportion of bees with detectable residues is relatively low (approximately 20%) in this data set. With this frequency the probability of no bees having detectable residues in a random sample of 5 bees is p = (1-0.20)5 = 0.327, which makes the finding of 1 such sample in 5 quite reasonable (Table 2). For 10 bees, p = 0.107. The proportion of bees with residues will vary in different circumstances but the variability of the results in this data set was caused by the number of bees in a subsample that contained detectable residues as well as the variation in the amount in each bee. With 20% of bees containing residues, the sample size should be at least 10 bees to avoid erroneous non-detections. In general, the distribution of residues among the bees in a colony is expected to become more uniform within hours due to trophallaxis9-11. The use of 100 bees from the brood area bees is therefore sufficient for quantitation of residues. These bees came from a healthy hive with no symptoms of impairment, indicating that the levels found were not harmful to the bees.

Sample stability – Neonicotinoids and viruses are stable in frozen bee samples4, 6. However, the stability of residues in dead bees collected in front of the hive at ambient temperature during the time before they were collected was not known. The stability of neonicotinoids at ambient temperature in a samples of bees was verified by analyzing subsamples from a field-collected sample known to contain significant levels of CLT and TMX at a series of times over 27 days. No significant degradation was detected.

Virology – Table 3 shows the distribution of viruses found in visibly impaired bees using the Quantigene method3. These bees had many of the same nonspecific symptoms as those reported for pesticides over- exposure12. Such bees were not always present during the assessments. The results show a variety of viruses, but DWV is most frequent and had the highest titer. In addition, 76.9% of black bees had detectable virus in this assay. The predominant virus was DWV even though none of the black bees had deformed wings. This shows that infection occurred after emergence as adults. Viruses were also frequently detected in the samples of brood area bees and

foragers showing no visible signs of disease. Energetics measurements have shown effects of DWV on performance of foragers that would not be apparent to the apiarist13.

Table 3 Virology – Impaired bees

Site	Date	ABPV	BQCV	CBPV	DWV	IAPV	KBV	SBV	VDV	No. of Viruses
1	At Plant	-	-	-	17423.4	+	-	-	201.1	3
1	Post Plant	-	-	38662.4	35970.3	+	-	5354.6	-	4
1	Midsummer	-	-	-	347.9	-	-	-	-	1
1	Fall	-	-	-	26128.7	-	-	1592.5	21634.5	3
2	At Plant	-	-	15583.2	148.2	-	-	+	-	3
2	Post Plant	-	-	-	-	5126.8	-	+	-	2
2	Midsummer	-	-	-	+	-	-	-	-	1
2	Fall	-	-	-	18573.2	18297.0	-	-	1621.5	3
2	Pre-plant 2015	-	-	-	-	-	-	-	-	0
3	Pre-Plant	-	-	-	259.5	-	-	-	-	1
3	Post Plant	-	-	-	44944.4	-	-	+	427.8	3
3	Midsummer	-	-	-	166.2	-	-	-	-	1
3	Midsummer	-	76.0	-	1479.8	-	-	-	-	2
3	Fall	-	-	-	8052.5	165.2	-	-	794.0	2
4	Pre-Plant	-	-	-	+	-	-	-	-	1
4	At Plant	-	-	-	124.4	+	-	-	-	2
4	Pre-plant 2015	-	-	-	+	8234.4	-	-	-	2
5	Pre-Plant	-	-	-	353.9	-	-	-	-	1
5	Post Plant	-	-	-	-	-	-	-	-	0
Frequency of Infection (%)		-	5.26	10.5	78.9	36.8	-	26.3	26.3	89.5
Total No. of samples=19										

Analytical results - The results of the analysis showed that the frequency of detection was below 35% in all sample types (Table 4). CLT was most frequently found followed by TMX, IMI and ACM.

Table 4 Analytical results for bees and hive materials

Sample Type	Total No. of Samples	TMX	CLT	TZNG	ACM	IMI	TMX	CLT	TZNG	ACM	IMI
Bees				**Frequency (%)**					**Maximum (ng/bee)**		
Brood Area Bees	143	1.4	1.4	0.0	2.8	0.7	(0.036)	(0.069)	0.0	0.46	0.097
Larvae	78	0.0	3.8	0.0	0.0	0.0	0.0	(0.064)	0.0	0.0	0.0
Foragers	95	10.5	11.6	0.0	0.0	0.0	0.11	0.674	0.0	0.0	0.0
Impaired Bees	20	5.0	20.0	5.0	0.0	0.0	0.046	0.428	0.2	0.0	0.0
Black Bees	14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dead Bees	89	5.5	34.1	18.7	0.0	0.0	0.077	0.66	0.32	0.0	0.0
LOD (100 mg/bee)							0.048	0.096	0.20	0.024	0.048
LOQ (100 mg/bee)							0.016	0.03	0.067	0.008	0.016
Hive materals				**Frequency (%)**					**Maximum (ng/g)**		
Capped Honey	140	10.7	3.6	0.0	12.1	0.7	1.2	0.95	0	8.2	0.165
Nectar	130	8.5	0.8	0.0	10.8	0.0	1.1	0.49	0.0	2.1	0.0
Pollen (Foragers)	101	23.8	23.8	4.0	9.9	9.9	20.2	45.5	2.8	5.3	2.2
Pollen (In-hive)	117	41.0	41.9	2.6	16.2	3.4	14.7	16.7	2.9	2.9	0.6
Wax	108	3.7	4.6	3.7	4.6	2.8	0.8	2.2	1.7	7.2	0.8
LOD							0.48	0.96	2.0	0.24	0.48
LOQ							0.16	0.32	0.67	0.08	0.16

Note: Values in brackets are below between LOD and LOQ

All results were below levels considered to be harmful[14]. The concentrations found were variable and the amounts found in bees were much lower than in hive materials, corresponding to less than 7 ng/g, than in hive materials, indicating rapid metabolism.

In summary, Over 90% of impaired bees had viruses, but 20% or less had any of the test compounds and only at low levels (<0.05 ng neonicotinoids /bee) (Table 3 and 4). 77% of black bees had viruses but none of the test compounds was detected in these bees. No neonicotinoids were detected in black bee samples (Table 4).

Aggregate risk: The risk of toxic effects from exposure to a mixture of compounds with a common mode of action such as the neonicotinoids can be estimated if the contributions of the components are converted to units of measure that can be summed. Toxicity Units (TU) are defined as the ratio of the dose (exposure) to a toxic endpoint such as the no effect level (NOEL)[15], assuming that interactions between compounds is insignificant[16]. This is the same as the sum of the risk ratios for the individual compounds, and the aggregate risk is given by:

$$\text{Aggregate TU} = \sum_{i=1}^{n} D_i / \text{NOEL}_i$$

Where: D_i = dose /concentration of the ith compound in the bee (ng/bee)

$NOEL_i$ = mortality NOEL of the ith compound (ng/bee)

n = number of compounds found in the sample

Trace values between the LOD and LOQ were used as reported

When this work was planned, mortality of bees was the effect of primary interest, and the NOEL values for mortality were available for the compounds of interest in the literature[14]. The maximum aggregate TU <1 (n=89). The risk to brood area bees and forager bees was not significantly different (Paired 1-sided T-test, n=89, p=0.44). This is not unexpected, given the rapid exchange of nectar among adult bees in the colony[9] and the provisioning of foragers from the colony[17-20]. However, Aggregate TU for larvae was significantly lower indicating that larvae are protected from exposure to the neonicotinoids (Paired 1-sided T-test, n=78, p=0.000255) [21] within the colony. This assessment can be updated as new endpoints become available.

Hive weight gain – The measurements of hive weight every 15 minutes provides a detailed, noninvasive and almost continuous measure of colony population and health, in addition to many detailed features as illustrated in Figure 2. Major events such as swarming, the start and end of honey flow or survival throughout the year are easily seen.

The net weight gain and loss through much of the annual cycle of colony life is highly variable, but the rapid weight gain the spring honey flow was sufficiently consistent to allow a test for an association between hive weight gain and the average aggregate TU. The results in Figure 3 show no adverse effect relationship. In fact there is a weak positive effect that is significant at the 10% level (Pearson's R 0.4959, n=14, p= 0.07).

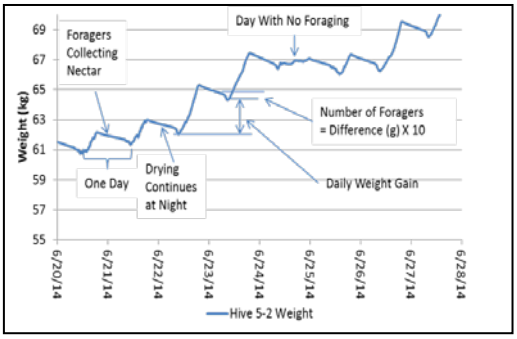

Figure 2: Hive weight from Beewatch scales: Example record from a healthy colony

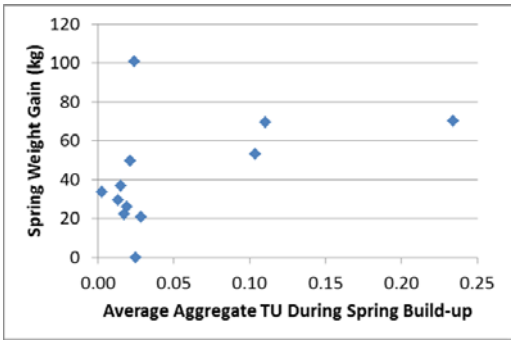

Figure 3: Weight gain vs. Aggregate TU

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References:

1. Purdy, J. R., Monitoring in-hive residues of neonicotinoids in relation to bee health status. Julius-Kühn-Archiv 2015, 450, 276.
2. Anastassiades, M., Lehotay, S. J., Stajnbaher, D., Schenck, F., Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. Journal of the Association of Official Analytical Chemists, International 2003, 86 (2), 412-431.
3. Affymetrix panomics products plex-2.0-assay: how-it-works. http://www.panomics.com/products/gene-expression/plex-2.0-assay/how-it-works (accessed Oct 3, 2015).
4. de Miranda, J. R., Bailey, L., Ball, B. V., Blanchard, P., Budge, G. E., Chejanovsky, N., Chen, Y.-P., Gauthier, L., Genersch, E., de Graaf, D. C., Ribière, M., Ryabov, E., De Smet, L., van der Steen, J. J. M., Standard methods for virus research in *Apis mellifera*. Journal of Apicultural Research 2013, 52 (4).
5. Human, H., Brodschneider, R., Dietemann, V., Dively, G., Ellis, J. D., Forsgren, E., Fries, I., Hatjina, F., Hu, F.-L., Jaffé, R., B., J. A., Köhler, A., Magyar, J. P., Özkýrým, A., Pirk, C. W. W., Rose, R., Strauss, U., Tanner, G., Tarpy, D. R., van der Steen, J. J. M., Vaudo, A., Vejsnæs, F., Jerzy Wilde, J., Williams, G. R., Zheng, H.-Q., Miscellaneous standard methods for Apis mellifera research in The COLOSS BEEBOOK, Volume I: Standard methods for Apis mellifera research. Dietemann, V. ; Ellis, J. D. and Neumann, P. (Eds). Journal of Apicultural Research 2013, 52 (4), http://dx.doi.org/10.3896/IBRA.1.52.4.10.
6. Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R., vanEngelsdorp, D., Pettis, J. S., High levels of miticides and agrochemicals in North American apiaries: Implications for honey bee health. PLoS One 2010, 5, e9754.
7. Chauzat, M.-P., Faucon, J.-P., Martel, A.-C., Lachaize, J., Cougoule, N., Aubert, M., A survey of pesticide residues in pollen loads collected by honey bees in France. J. Econ. Entomol. 2006, 99 (2), 253-262.
8. Chauzat, M., Martel, A., Cougoule, N., Porta, P., Lachaize, J., Zeggane, S., Aubert, M., Carpentier, P., Faucon, J., An assessment of honeybee colony matrices, *Apis mellifera* (Hymenoptera: Apidae) to monitor pesticide presence in continental France. Environ. Toxicol. Chem. 2011, 30 (1), 103-111.
9. Pankiw, T., Nelson, M., Page, R. E., Fondrk, M. K., The communal crop: modulation of sucrose response thresholds of pre-foraging honey bees with incoming nectar quality. Behav. Ecol. Sociobiol. 2004, 55, 286-292.
10. Goyret, J., Farina, W. M., Trophallactic chains in honeybees: a quantitative approach of the nectar circulation amongst workers Apidologie 2005, 36 (4), 595-600.
11. DeGrandi-Hoffman, G., Hagler, J., The flow of incoming nectar through a honey bee (*Apis mellifera* L.) colony as revealed by a protein marker. Insectes Soc. 2000, 47, 302-306.
12. Kozak, P., How to Identify and Report Suspected Bee Poisonings. Ontario Bee Journal 2014, 33 (3), 12-13.
13. Wells, T., Wolf, S., Nicholls, E.,; Groll, H., Lim, K. S., Clark, S. J., Swain, J., Osborne, J. L., Haughton, A. J., Flight performance of actively foraging honey bees is reduced by a common pathogen. Environ Microbiol Rep. 2016 8(5), 728-737.
14. PMRA, Evaluation of Canadian Bee Mortalities Coinciding with Corn Planting in Spring 2012. Health Canada 2013.
15. Faust, M., Altenburger, R., Boedeker, W., Grimme, L. H., Additive effects of herbicide combinations on aquatic non-target organisms. Sci. Total Environ. 1993, Supplement 2, 941-951.
16. Thompson, H. M. Interaction between pesticides and other factors in effects on bees; 2012; p 204.
17. Beutler, R., Zeit und Raum im Leben der Sammelbiene. Die Naturwissenschaften 1950, 37, 102-106.
18. Brandstetter, M., Crailsheim, K., Heran, H., Provisioning of food in the honeybee before foraging. In BIONA-report 6, Akad. Wiss. u. Lit. , Nachtigall, W., Ed. G. Fischer: Mainz, Stuttgart, 1988; pp 129-148.
19. Visscher, P. K., Crailsheim, K., Sherman, G., How do honeybees (*Apis mellifera*) fuel their water foraging flights? J. Insect Physiol. 1996, 42, 1089-1094.
20. Crailsheim, K., Trophallactic interactions in the adult honeybee (*Apis mellifera* L.). Apidologie 1998, 29 (1-2), 97-112.
21. Purdy, J., Concentrations and distribution of neonicotinoid residues in honeybees (*Apis mellifera*) in Ontario, Canada. Poster No. AGRO 617. In American Chemical Society Joint Meeting with the International Union of Pure and Applied Chemistry, San Francisco, 2014.