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Detection of the desiccant and plant growth regulator chlormequat in honeybees and comb pollen

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ABSTRACT: We here describe the results of the investigation of a honeybee hive that exhibited symptoms of poisoning in the July 2013. During the summer, only a few pesticides such as desiccants, typically quaternary ammonium (quat) and glyphosate formulations, are applied. We therefore analysed samples of not only a wide range of pesticides, but also desiccants. Additionally, we analysed dying honeybees, already dead individuals and comb pollen from the case hive. The LC-MS/MS (triple quadrupole) analysis of glyphosate-based quaternary ammonium pesticides (quats) and a wide range of 148 other pesticides allowed identification of 23 different pesticides in the samples: (I) two quats, (II) twelve fungicides, (III) four insecticides, (IV) four herbicides and (V) one rodenticide. The plant growth regulator chlormequat was the pesticide identified in the highest amounts and was present in all of the analysed samples (bees and pollen). However, it is regarded as being practically non-toxic to honeybees. In summary, this manuscript describes the detection of quat in honeybees and pollen.

Keywords: desiccant; pesticide; quaternary ammonium pesticides; *Apis mellifera*

Honeybees, primarily *Apis mellifera* Linnaeus, remain the most important and economically valuable pollinators of agricultural crops worldwide (Klein et al. 2007). Recently, disturbing declines have been documented in honeybee populations in many European and North American countries, particularly after wintering of the hives (van der Zee et al. 2012; vanEngelsdorp et al. 2012; Spleen et al. 2013). Altogether, 61 probable contributing factors have been implicated in pollinator losses, and these include *Varroa destructor* mites, poor nutrition, exposure to pesticides and agrochemicals and various pathogens and pests (vanEngelsdorp et al. 2009). Recent studies have primarily linked honeybee colony losses with pesticide exposure, because the natural environment is highly contaminated with a large array of pesticides and other chemicals (Pettis et al. 2013). Currently, the main culprits are believed to be newly developed systemic insecticides, such as neonicotinoids and fipronil, that entered the market in the mid-1990s (Aliouane et al. 2009; Mommaerts et al. 2010; Blacquiere et al. 2012); however, the pesticides affecting bees include not only insecticides but also fungicides and herbicides (Johnson et al. 2010; Maini et al. 2010; Pettis et al. 2013). Recently, sub-lethal doses of glyphosate were reported to negatively influence honeybee navigation (Balbuena et al. 2015), and in another study, chlormequat poisoning was found to resemble anticholinesterase toxicity (Nisse et al. 2015). However, previous studies did not consider desiccants and plant growth regulators, such as quaternary ammonium pesticides (quats), to be contaminants of honeybee hives.

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Desiccants have several practical uses in crop production. Pre-harvest desiccation using quats or glyphosates reduces the weed seed bank and results in a higher-quality product by facilitating the harvest (Daltro et al. 2010), assisting in harvest planning (Veiga et al. 2007) and preserving seed productivity and quality (Lacerda et al. 2005). The negative effects of desiccants include residues in weeds (Vahl et al. 1998) and in the environment (Zhao et al. 2013) as well as possible impacts on non-target organisms. This study is a case report of a suspected poisoned honeybee hive located at the Bee Research Institute at Dol; it was the only hive out of 50 nearby surrounding hives and at least 200 hives within flying range to exhibit poisoning symptoms. Wide-ranging pesticide contamination was documented in individual honeybees and pooled samples, and the pesticide detected in the bees and pollen in the highest abundance was chlormequat. These compounds can influence bees at sub-lethal doses and can act synergistically with other pesticides as well as any other xenobiotics.

MATERIAL AND METHODS

Biological samples. The honeybee samples used in this study were collected at the Bee Research Institute at Dol in the Czech Republic on the 17th–18th of July 2013 from an *Apis mellifera carnica* colony that exhibited sudden worker death directly outside of the hive (Figure 1). The hive was the sixth of nine hives in a row. In addition, 50 hives were located nearby, and at least 200 hives owned by the Bee Research Institute at Dol were located within

Figure 1. Photographic documentation of the sudden inexplicable dying of honeybee workers outside the hive: (**A**) The case hive was the sixth in a series of nine hives and is indicated by the arrow; (**B**) dead and dying honeybees outside the hive; (**C**) detailed view of dead and dying honeybees outside the case hive; (**D**) closer detail of dead and dying honeybees outside the case hive; the arrow indicates a dying individual

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flying range of the case hive. However, none of the other hives exhibited symptoms similar to those of the case hive during the collection year. Bees were first observed to be dying outside the hive three weeks before sample collection and accumulated during the days of sample collection. Later, no bee decline was observed, and the colony appeared in good condition and was overwintered. Outside the hive, tweezers were used to collect deceased honeybees (Figures 1B and 1C) as well as twitching and dying (still alive) individuals (Figure 1D); the dying and dead honeybees were on their backs with their probosces extended. Comb pollen was also collected from the hive. Figure 2 outlines the strategies used for sample collection, sample processing and pesticide analysis. After collection, the bee samples were stored on dry ice for transport to the laboratory, where they were directly processed and subjected to LC-MS/MS analysis within a week.

Sample processing of dying individuals. Twelve dying individual honeybees (Figure 1D) were selected for pesticide analyses. The honeybees were weighed and surface-extracted after being dipped in 1.2 ml of acetonitrile (LC-MS grade; Cat No. A/06038/17; Fischer Scientific; Waltham, USA) for 15 min and vortexed for 2 × 30 s in parallel (MS1 Minishaker, IKA, Brazil). The individuals were removed to a 2-ml glass Potter-Elvehjem homogeniser (Art. No. 6302; Kartell Labware Division, Noviglio, Italy), and the surface extracts from the 12 honeybees were mixed together to obtain one pooled sample, which was filtered through a 0.45-µm regenerated cellulose filter (OmniPeak; Teknokroma, Spain). We did not test whether the surface dipping in ACN extracted inner pesticides, but the highly divergent results between the surface and the body extracts suggest that this is not the case (or only to a very limited degree; see the results). Next, 1.2 ml of acetonitrile were added to each honeybee in the 2-ml Potter-Elvehjem homogeniser, and the samples were homogenised using a drilling machine. The homogenate was transferred to 1.5-ml centrifuge tubes and centrifuged at 20 000 × *g* and 4 °C for 20 min in an MR 23i centrifuge (Jouan Industries, France). Pure acetonitrile samples were prepared as controls. The samples were transferred to brown 1.5-ml glass vials with screw caps (Cat No. 5182, Agilent Technologies, Germany) and stored in a rack at –30 °C until use.

Processing of dead individuals and comb pollen samples. In total, 158 dead honeybees were divided into two groups of 79 individuals. The dead bees were not surface homogenised because their structures may have been disturbed by decomposition processes; thus, the possible surface exposure of the dying bees to pesticides was analysed. The samples were homogenized in 0.25 ml of acetonitrile per individual (19.75 ml per 79 individuals) in 100-ml glass Potter-Elvehjem homogenisers (Kavalier, Sazava, Czech Republic).

A drilling machine was used to homogenise the samples. The homogenate was transferred to a 50-ml centrifuge tube (Orange Scientific, Braine-l'Alleud, Belgium) and centrifuged at 10 000 × *g* and 4 °C for 15 min in an MR 23i centrifuge (Jouan Industries, France). Then, the supernatants were

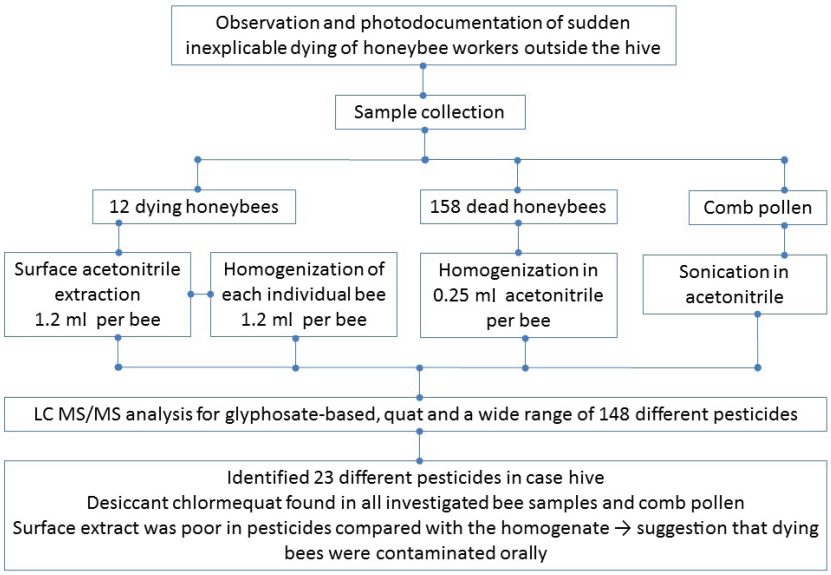

Figure 2. Schematic workflow of the strategies used for the sample collection, sample processing and pesticide analysis

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collected, pooled and centrifuged again in 1-ml centrifuge tubes at 20 000 × *g* and 4 °C for 20 min in an MR 23i centrifuge. Following centrifugation, the supernatant was transferred to a Luer-lock syringe and filtered through a 0.45-µm regenerated cellulose filter (OmniPeak; Teknokroma, Spain). The pollen extract was prepared as follows: pollen was mixed with 2 ml of acetonitrile, sonicated for 30 min and then centrifuged for 5 min at 11 000 × *g*. As controls, pure acetonitrile samples were prepared. The samples were divided into brown 1.5-ml glass vials with screw caps (Cat No. 5182, Agilent Technologies, Germany) and stored in a rack at –30 °C until use.

Pesticide analysis. Analysis of honeybee samples and pollen collected from the hive was similar to that of glyphosate-based and quat pesticides as well as a wide range of 148 other pesticides, and all the analyses are routinely used to detect pesticides at ALS Czech Republic, Prague (http://www.alsglobal.eu/). Acetonitrile extracts generally do not require additional clean-up steps; thus, the quats and 148 pesticides were analysed using direct injection with no sample pre-treatment. The method of analysis for glyphosate-based pesticides and their metabolites (for the list with specifications, see Table S1 in electronic supplementary material (ESM); for the supplementary material see the electronic version) followed the Applied Biosystems No. 114AP51-01 application guide, and the analysis was performed using an LC (1100 Series, Agilent, Santa Clara, USA)-MS/MS system (API 4000, triple quadrupole, Applied Biosystems, Carlsbad, USA). The samples used for glyphosate analysis were first derivatised and further concentrated using SPE columns (Cat. No. 8B-S100-FCH, Strata X 33 µm, 85A, 200 mg/6 ml Phenomenex, Torrance, USA). Quats (for the list with specifications, see Table S2 in ESM) were analysed using an LC-MS/MS system (Acquity I class/Xevo TQ-S, triple quadrupole, Waters, Milford, USA) according to the Applied Biosystems 114AP43-01 application guide. The remaining 148 pesticides (for the list with specifications, see Table S3 in ESM) were determined using the same LC-MS/MS conditions as the glyphosate pesticides and following the Applied Biosystems 114AP43-01 application guide.

Evaluation of pesticide toxicity. To evaluate the toxicity of the identified pesticides, we searched the available data in the following databases (Tables S4–S7 in ESM): (I) the Pesticide Action Network (PAN; http://www.pesticideinfo.org/); (II) the United States Environmental Protection Agency (EPA); (III) the Food and Agriculture Organization of the United Nations (FAO); and (IV) the European Commission – Health and Consumer Protection Directorate-General (EC).

RESULTS

Identification of pesticides

All the samples of individual honeybees (see Table 1; 1A–12A, 13SE) and comb pollen (see Table 1; P1) as well as the pool of dead honeybees (see Table 1; 13 WH) contained pesticides. A total of 23 pesticides were identified in the samples (see Table 1; samples P1, 1A–12A, 13SE, 13 WH). The dominant, most frequent and most abundant pesticide found in the samples was the quat chlormequat; this pesticide was identified in all the honeybee samples as well as the pollen sample. The second most frequent pesticide among the samples was the fungicide picoxystrobin. We identified two quats (paraquat and chlormequat), 12 different fungicides (picoxystrobin, propiconazole, azoxystrobin, carbendazim, fenhexamid, flusilazole, hexaconazole, imazalil, kresoxim-methyl, metconazole, prochloraz, and tebuconazole), four different insecticides (acetamiprid, dichlorvos, fensulfothion, and imidacloprid), four herbicides (propachlor, dichlormid, linuron, and metazachlor) and one rodenticide (warfarin). Dichlorvos was only detected in the surface extract of the honeybees, whereas acetamiprid, fenhexamid, flusilazole, hexaconazole, imidacloprid and metconazole were identified in the dead honeybee extract (see Table 1; 13 WH) but not in the dying individuals. In contrast, propiconazole, dichlormid, imazalil, linuron and prochloraz were exclusively identified in individual honeybees (one individual), and metazachlor was only identified in comb pollen.

Evaluation of pesticide toxicity

The data showing the toxicity level of the identified pesticides that were found in the PAN, EPA, FAO and EC databases are summarised in the Supplementary material (Tables S4–S7 in ESM). Most of the identified pesticides are considered practically non-toxic,

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Table 1. Pesticides found in samples reported as ng per honeybee individual and ng per g of comb pollen

	Pesticide	P1	1A	2A	3A	4A	5A	6A	7A	8A	9A	10A	11A	12A	13SE	13 WH
Q	Paraquat	–	–	4.84	–	5.16	–	–	5.42	–	–	4.20	–	2.41	–	–
Q	Chlormequat	7.692	55.32	216	4.56	38.28	21.12	34.68	182.4	32.28	16.08	202.44	271.44	38.04	0.31	0.38
F	Picoxystrobin	–	0.12	0.58	–	0.06	0.14	0.06	1.1	0.09	0.11	0.86	0.4	0.36	–	0.05
H	Propachlor	–	0.15	–	–	–	–	–	–	–	–	0.07	–	–	–	0.01
F	Propiconazole	–	–	–	–	–	–	–	–	–	0.07	–	–	–	–	–
F	Azoxystrobin	–	–	–	–	–	–	–	–	–	–	–	–	–	0.02	0.11
I	Acetamiprid	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.02
F	Carbendazim	–	–	0.18	–	–	–	–	0.09	–	–	–	0.07	–	–	0.02
H	Dichlormid	–	–	1.25	–	–	–	–	–	–	–	–	–	–	–	–
I	Dichlorvos	–	–	–	–	–	–	–	–	–	–	–	–	–	0.06	–
F	Fenhexamid	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2.88
I, N	Fensulfothion	–	–	0.31	–	0.12	–	–	3.65	–	–	0.55	1.13	1.09	–	0.19
F	Flusilazole	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.01
F	Hexaconazole	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.38
F	Imazalil	–	–	–	–	0.17	–	–	–	–	–	–	–	–	–	–
I	Imidacloprid	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.24
F	Kresoxim-methyl	–	–	92.4	–	7.32	–	–	57.96	–	–	33.12	19.68	22.8	–	1.98
H	Linuron	–	–	0.14	–	–	–	–	–	–	–	–	–	–	–	–
F	Metconazole	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.02
F	Prochloraz	–	–	–	–	–	–	–	–	–	–	0.1	–	–	–	–
F	Tebuconazole	–	–	0.97	–	0.16	–	–	2.06	–	–	1.07	0.54	0.36	–	0.13
H	Metazachlor	0.335	–	–	–	–	–	–	–	–	–	–	–	–	–	–
R	Warfarin	–	–	0.97	–	–	–	–	0.97	–	–	2.47	2.39	0.93	–	–

1A–12A = dying individual honeybees, 13SE = mixed honeybee surface extract from 1A–12A, 13 WH = dead honeybee whole body homogenate of 158 individuals, F = fungicide, H = herbicide, I = insecticide, Q = quat (plant growth regulator), N = nematicide, P1 = pollen sample

non-toxic or slightly toxic to honeybees; only dichlorvos, imidacloprid and fensulfothion are classified as highly toxic to honeybees. Acetamiprid, paraquat and kresoxim-methyl are classified as slightly to moderately toxic, and data related to the toxicity of warfarin and hexaconazole for honeybees were not found in the databases.

DISCUSSION

The results of this case study highlight the importance of studying pesticide contamination in the environment and the poisoning of major crop pollinators, namely, honeybees. In total, we identified 23 different pesticides in the honeybees and comb pollen collected from the case hive. Due to the wide range of pesticides identified, the honeybees were gradually contaminated, and the terminal contamination was likely caused by the desiccant chlormequat, which would be in line with fact that only a few pesticides, such as desiccants (quats, glyphosates), are applied in July. The present work is also important given the methodology applied to extract and analyse the pesticides from the surface (see Table 1; 13SH) and body homogenate (see Table 1; 1A–12A) of the individual honeybees; this analysis allowed us to determine whether the bees were contaminated orally or by spraying. The fact that the surface extract yielded a very different amount of pesticides than the body extract suggests that pesticides were not extracted from the body by surface extraction, or only to a very limited extent, because the amount of chlormequat in the two extracts differed by approximately two orders of magnitude. Moreover, the finding that the surface extract contained low levels of pesticides compared with the homogenate suggests that the

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bees were primarily orally contaminated by the pesticides, which is also supported by the detection of chlormequat in the comb pollen.

Despite the wide array of pesticides identified in the investigated samples, the majority of honeybee mortality could be attributed to chlormequat, a desiccant and plant growth regulator (PGR) whose effects resemble that of anticholinesterase insecticide poisoning (Nisse et al. 2015); this pesticide was identified in the highest amounts in the samples. The dying and dead honeybees from our case hive were found on their backs with their probosces extended, which is a manifestation of nerve poisoning in bees (Fairbrother et al. 2014). However, these signs may have been due not only to chlormequat alone, but also might have been a result of its combination with the other identified pesticides. Acute and oral contact with chlormequat is considered to be practically non-toxic to honeybees, and its LD_{50} is similar to that of glyphosate, > 100 µg per bee (EPA 2007). However, it was recently shown that the sub-lethal dose of 0.5 µg of glyphosate per bee influences honeybee navigation (Balbuena et al. 2015), and it is also necessary to consider that the pesticide doses we detected in the bees were presumably low because pesticides undergo metabolic changes in organisms. This phenomenon is more likely to have occurred in the dead than the dying individuals and is in line with the relatively low amount of chlormequat found in the dead bees. Another quat that we detected in some of the samples of the dying bees was paraquat, which is important because this pesticide has been banned in the EU since 2007 (Court of Justice 2007, http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:62004TJ0229). However, while paraquat is considered extremely hazardous to humans (Gawarammana and Buckley 2011), in bees it is considered practically non-toxic or only slightly toxic (Tables S4–S7 in ESM).

Relatively large amounts of the fungicides kresoxim-methyl and fenhexamid were also identified, and these fungicides together with the ten other identified fungicides could have contributed to the decline of the bee colony. However, only 1.98 ng of kresoxim-methyl per bee and 2.88 ng of fenhexamid per bee were found in the dead bee samples (see Table 1; 13 WH); these levels are not lethal because these substances are considered to have low toxicity (LD_{50} > 10 µg per bee for fenhexamid and > 100 µg for kresoxim-methyl; see Tables S4–S7 in ESM) in honeybees. The highest concentration of kresoxim-methyl, 92.40 ng per bee, was found in sample 2A of a dying individual honeybee, but this was still well below the lethal dose. Similar to the study by Pettis et al. (2013), our results indicate the importance of fungicides in bee decline given that a total of 12 different fungicides were identified in individual honeybees. In various previous studies, the acute toxicity (oral and contact in the laboratory) of imidacloprid in *A. mellifera* ranged from 0.2 ng to 154 ng per bee (Blacquiere et al. 2012), and in our study, the concentration was 0.24 ng per dead bee. Thus, this concentration of imidacloprid could have contributed to the honeybee decline, but we were unable to identify this neonicotinoid in the dying individuals (see Table 1; 1A–12A), which may have been due to a lower detection limit in the dying bee samples (the dead bee samples were homogenised in 0.25 ml of acetonitrile per bee while the dying bees were homogenised in 1.2 ml of acetonitrile). However, a pesticide cocktail, as opposed to a single pesticide, was most likely the cause of the bee decline. Interestingly, we also identified warfarin, a rodenticide, in individual bees, and to the best of our knowledge, this manuscript constitutes the first report of this type of pesticide in honeybee samples. This result implies that the honeybees had eaten the rodenticide, which indicates that contamination of bees by other xenobiotics, such as rodenticides, is possible.

Several studies have found pesticide residues in both floral nectar and pollen (Krischik et al. 2007; Choudhary and Sharma 2008; Dively and Kamel 2012). An analysis of pollen carried by foraging honeybees returning to their hive was recently performed, and the estimated total of 35 different pesticides in the samples implies that the bees were exposed to a highly damaging and variable pesticide cocktail (Pettis et al. 2013). We analysed individual honeybees and pollen from one case hive and successfully identified 23 different pesticides in the samples; this diversity of pesticides in one hive illustrates the extent of environmental damage by contamination with different human-produced xenobiotics. The problem of pesticide contamination in the environment is complex, and there is an urgent need to investigate all the xenobiotics, including pesticides as well as their metabolites, in the environment. This case study showed that quat pesticides are also important, particularly in the season when desiccants are applied. Validated analytical tools are needed to identify the wide

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range of environmental contaminants in bees and bee matrices.

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