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Neonicotinoid insecticides in pollen, honey and adult bees in colonies of the European honey bee (*Apis mellifera* L.) in Egypt

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Abstract Honeybee losses have been attributed to multiple stressors and factors including the neonicotinoid insecticides (NIs). Much of the study of hive contamination has been focused upon temperate regions such as Europe, Canada and the United States. This study looks for the first time at honey, pollen and bees collected from across the Nile Delta in Egypt in both the spring and summer planting season of 2013. There is limited information upon the frequency of use of NIs in Egypt but the ratio of positive identification and concentrations of NIs are comparable to other regions. Metabolites of NIs were also monitored but given the low detection frequency, no link between matrices was possible in the study. Using a simple hazard assessment based upon published LD_{50} values for individual neonicotinoids upon the foraging and brood workers it was found that there was a potential risk to brood workers if the lowest reported LD_{50} was compared to the sum of the maximum NI concentrations. For non-lethal exposure there was significant risk at the worst case to brood bees but actual exposure effects are dependant upon the genetics and conditions of the Egyptian honeybee subspecies that remain to be determined.

Keywords Hazard assessment · Agriculture · Honey bees · Metabolites · Imidacloprid · LD_{50}

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Introduction

Insects, such as the Western honeybee (*Apis mellifera*), are essential for pollination of wild flowers and agricultural crops (Ollerton et al. 2011). Though only 35% of food crops based upon mass grown today require pollinators the pollinator-independent plants are made up predominantly of 28 species while pollinator assisted plants are dominated by 85 plant species (Klein et al. 2007). Any loss of pollinators will threaten human dietary diversity, food production security and the biodiversity of wild plant species (Corbet et al. 1991; Klein et al. 2007). Moreover, the global dependence of pollinators is not homogeneous with some regions, such as the Mediterranean being far more reliant on pollinators than other countries (Lautenbach et al. 2012). These pollinated crops also provide many essential nutrients

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and minerals necessary for human health and the loss would cause some 29 million lost years of health annually based upon many factors including for example deficiencies of vitamin A and iron (Chaplin-Kramer et al. 2014).

Today in modern agriculture, there are many insects that are farmed for crop pollination including bumblebees (*Bombus impatiens*) for plants like tomatoes and the western honeybee for a wide range of plants including apples, oilseed rape and almonds. Of all the managed pollinators the western honeybee represents ~90% of all managed pollinators, *with* more than 44 subspecies recognized worldwide (Engel 1999). Given the reliance of the honeybee in some regions, any decline in the species can impact heavily on food production. Between 2007–2008 the United States beekeeping industry reported over winter loss of some 80 thousand hives of the western honeybee, this is ~1/3 of the total commercial hives in the US (vanEngelsdorp et al. 2009). The importance of commercial bees in the can be seen in the US almond industry, where 80% of the world's almonds are grown. This production requires ~85% of the US commercial hives to pollinate and any bee decline would affect the industry (NASS 2016). In the EU it is estimated that a deficit of 13 million colonies exist to maximize pollination within Europe. However, it is not just the *Apis* species in decline, the wild bumblebee is also in decline with 13 species in the EU alone being classified as extinct in 1 or more country (Goulson et al. 2008).

There are a number of stressors that can adversely affect populations of both wild and domesticated honeybees. These include the ectoparasitic mite (*Varroa destructor*) and Israeli acute paralysis virus (IAVP) that is considered a major factor in the colony collapse disorder (CCD) of the 2000s (Chen et al. 2014; Genersch et al. 2010). However, the interplay of plant protection products (PPP) and pathogens has been observed to cause greater impact than exposure either stressor alone (Gregory et al. 2005; Navajas et al. 2008; Yang and Cox-Foster 2007). Other factors including changes in climate, crops and the diversity of PPP routinely used have also been implicated in bee heath decline (Fairbrother et al. 2014; Genersch et al. 2010). Toxic assessments such as the LD_{50} are valuable tools in the assessment of adverse effects however, their scope is limited. With hive communities, the effects of non-lethal toxicity may play a role as great as that of lethality, with exposure to some factors affecting cognitive function (Decourtye et al. 2004a, b). Given mankind's reliance on the honeybee it is essential that we understand regional and global exposure to stressors.

Developed in the 1980s neonicotinoid insecticides (NIs) first came into use at the beginning of the 1990s with introduction of Imidacloprid (Kollmeyer et al. 1999). The mode of action of these compounds is to bind to the nicotine acetylcholine receptors (nAChRs), acting agonistically, causing paralysis and death. NIs bind more strongly to nAChRs of invertebrates, making them more toxic at lower doses than other compounds, with LD_{50} values as small as 18 ng bee^{-1} (2.5–44) for clothianidin and 11.8 ng bee^{-1} (3.5–30) for thiamethoxam (Table 1). If compared to legacy insecticides, such as DDT, where topical application LD_{50} values were in the µg bee^{-1} range, NIs are substantially more of a risk to bee health. Actual LD_{50} values for NIs vary among individual bees, source of NI, temperature and

Table 1 LD_{50} calculations were averaged based upon the values for oral exposure listed in the following publications: Laurino et al. (2013), EU Commission (2004), Suchail et al. (2001), Kamel (2010), Bonmatin et al. (2015), Nauen et al. (2001), Schmuck et al. (2001), Suchail et al. (2001), DEFRA (2007), DEFRA (2009), Iwasa et al. (2004), Decourtye and Devillers (2010), Elbert et al. (2008) and the USEPA pesticide and Ecotoxicology Database accessed 2017

Compounds	LD_{50}			Sub-lethal concentrations		
	Average ng bee^{-1}	Min	Max	Average	High	Low
Parent compounds						
Acetamiprid	10,140.0	7070.0	14,530.0	606.5	810.0	403.0
Clothianidin	18.0	2.5	44.0	20.5	40.0	1.0
Imidacloprid	120.0	3.7	490.0	2.7	3.9	1.5
Thiamethoxam	11.8	3.5	30.0	3.2	5.0	1.3
Dinotefuran	26.8	1.7	75.0	116.5	230.0	3.0
Metabolite compounds						
I-Olefin	32.0	28.0	36.0			
I-5-Hydroxy	190.0	153.0	258.0	139.5	159.0	120.0
I-Desnitro olefin	524.5	49.0	1000.0			
I-Desnitro–HCl	1000.0	1000.0	1000.0			
i-6-Chloronicotinic acid	608,000.0	1000.0	1215,000.0			

For non-lethal effects publications by Henry et al. (2012), Lu et al. (2014), Decourtye et al. (2004a,b, 2003), and the USEPA pesticide and Ecotoxicology Database accessed 2017 were used. Metabolites of imidacloprid (I) were also investigated based on frequency of determination in samples

other environmental and physical factors (Bonmatin et al. 2015; Decourtye and Devillers 2010; Iwasa et al. 2004; Laurino et al. 2013; Rinkevich et al. 2015).

Neonicotinoid insecticides have been subject to increasing investigation as a co-factor in pollinator losses. Since the early 2000s this class of insecticides has increased in use with approximately 1/3 of all insecticide treatments in 2010 being NIs (~20,000 tonnes of active ingredient; (Bonmatin et al. 2015)). There exist many benefits to the use of NIs they are potent at lower concentrations than other insecticides so need less active ingredient to be effective while being less toxic to fish and other vertebrates. However as they are they are water soluble and persistent such that they may be of risk to the environment (Goulson 2013). However, due to their systemic properties NIs require less foliar spraying compared to organophosphorus pesticides, such as pyrethroids. When applied to bee feed in colonies, colony collapse or pre collapse behavior has been observed for selected NIs (Lu et al. 2014; Yamada et al. 2012).

Egypt and particularly the Nile Delta, is considered the most important region for bees and honey production in the Middle East and North Africa with an estimated 1.3 million hives (Al Naggar et al. 2015a). There have been reports of colony losses with no definite cause (Hassan 2009), however, much of the beekeeping is performed by amateurs in the region and they often own single small box hives and destroy the hives over winter (Al Nagger et al. 2017, (submitted)). In Egypt, it is estimated that half of the population is involved in the agricultural industry with the major crops being cotton, clover, maize, rice, wheat, soybeans, sugar cane, sugar beet, grapes, oranges, potatoes. Cotton does not require the bee for pollination but it improves the quality of the cotton produced (Cunningham 2015), while maize, grapes, oranges, clover and potatoes all benefit from bees (Sammataro and Avitabile 1998). Root knot nematodes are of concern for many Egyptian plants, such as sugar beet and corn. NIs are a common crop protection product used in protection from nematodes (Elbert et al. 2008).

There is limited information on the volume of NIs used in Egypt, however, it was estimated that >30% of insecticide treatments have NIs as the active ingredient (Malhat et al. 2014). In 2013, 8677.05 tonnes of active ingredient was used in 148 formulations equating to ~2600 tonnes of NI used (Al Nagger et al. 2017, (submitted)). In a study of human contamination in Egypt, blood was tested for thiamethoxam and acetamiprid used by 29 and 26% of Egyptian farmers, respectively, but nothing of the parent compound was detected (Shalaby et al. 2012). As with other insecticides, residues of the parent compounds in blood are not always detected due to metabolism. In the case of acetamiprid, it appears that only the N-desmethyl-acetamiprid metabolite can be found in patients exposed to this insecticide (Taira et al. 2013). The same most likely applies to other NIs in human exposure.

The objective of this study was to quantify a range of neonicotinoids and their metabolites in bees, pollen and honey collected from 15 locations within the Nile Delta region during spring and summer of 2013. Hazards posed by dietary intake of NIs to dietary intake of forager and nurse bees were assessed by use of a risk assessment.

Methods

Standards, chemicals and reagents

Solvents; methanol (MeOH), Acetonitrile (ACN), and water were of analytical grade and purchased from EMD chemicals (Gibbstown, NJ, USA). NI standards, mass-labelled NIs (clothianidin, imidaclopid, thiamethoxam, thiacloprid, acetamiprid, nitenpyram, Imidacloprid (D^3) and Clothanidin (D^4)) and flonicamid. Sample extraction/cleanup materials magnesium sulphate ($MgSO_4$), sodium chloride (NaCl), trisodium citrate, ($Na_3C_6H_5O_7$), primary secondary amine (PSA), and disodium citrate, ($Na_2HC_6H_5O_7$), were purchased from Sigma Aldrich, Ontario, CA. Polypropylene falcon tubes (15 and 50 mL; tubes) were purchased from Fisher Scientific (Ontario, Canada).

Sample collection and handling

Within the Nile Delta flowering plants of medicinal, aromatic and ornamental use are most commonly pollinated by the honeybee (35.2% of total plants), followed by vegetables (34.1%), fruits (21.9%) and field crops (8.8%) (Abou-Shaara 2014). Honey is harvested three times per year, once in April after the citrus season, in June after spring clover season, and in September after the cotton season. The Citrus season honey harvest may be very small or not always collected so in this study the clover (spring) and cotton (summer) seasons were sampled. Three hives were selected at random, from each of the 15 professionally managed apiaries within the five primary governances of the Nile River Delta (Fig. 1). However, precise data on hive location to potential NI sources is not known in this study.

Honey was sampled directly off an open comb into 50 mL falcon tubes. Pollen was collected by cutting with a disposable plastic knife a 6 cm^2 piece of comb, containing stored pollen which was placed into 15 mL tubes. Worker bees were collected in polyethylene bags from the wall farthest from the entrance, as these bees tend to be older and more uniform in age than the rest of the hive (Al Naggar et al. 2013, 2015a, b). In total 45 samples were taken of each sample matrix. Samples were stored with ice packs in a cooler and initially stored at Tanta University in Egypt in a

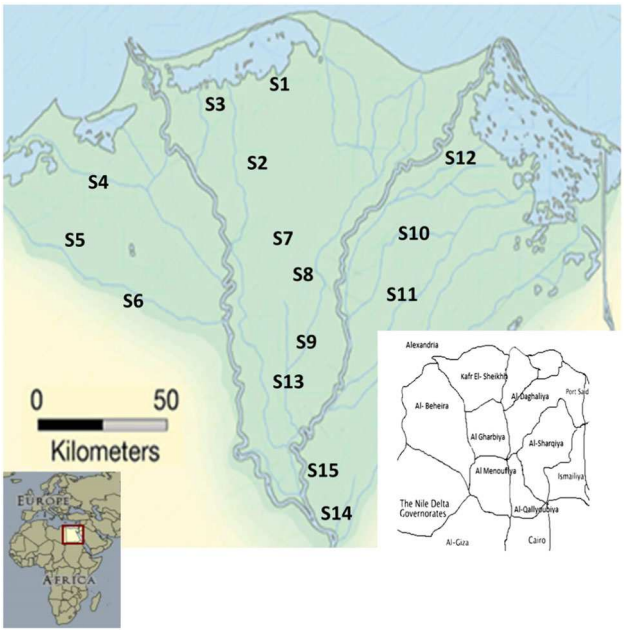

Fig. 1 Sampling locations within the Nile River Delta region of Egypt

freezer at −20 °C before transport to Saskatoon, SK, Canada, and stored at −20 °C. During transport to Canada, samples were stored in a thermal bag with ice packs with travel time of 20 h.

Extraction and cleanup

Extraction was performed using a modified QuEChRS method and NIs were identified and quantified by use of an HPLC–MS/MS method that has been described previously (Codling et al. 2016), with a slight modification in final supernatant volume (4 mL in this study compared to 6 mL), and quantification included additional metabolites. In brief, 5 g of honey or 2 g of pollen were weighed out into a 50 mL tube, fortified with D^3-Imidacloprid surrogate standard (SS). For bees, 5 g wet mass was homogenized with 3 g of $NaSO_4$ in a glass pestle and mortar before being added to a 50 mL tube with SS. Ten milliliters of nanopure water was added and the sample shaken for 20 min (min) at 2000 rpm, using a bench shaker (Heidolph MultiReax, Germany), 10 mL of ACN was then added and again shaken for 20 min. EN extraction salts (4 g $MgSO_4$, 1 g NaCl, 1 g $Na_3C_6H_5O_7$ and 0.5 g $Na_2HC_6H_5O_7$) prepared were added to each tube and shaken for a further 20 min.

Samples were centrifuged at 3000×*g* for 5 min, and 6 mL of ACN pipetted off to a 15 mL tube with 2 g $MgSO_4$ and 300 mg of PSA. This was shaken for 20 min, centrifuged for 5 min, and 4 mL of the supernatant was passed through a syringe filter (13 mm ⌀, 2 µm nylon syringe filter), to a clean 15 mL tube and taken to dryness under N_2. Samples were reconstituted in 200 µL ACN containing 50 ng mL^{-1} IS (D^4-Clothanidin), and analyzed by LC–MS/MS.

Analysis was by HPLC (Agilent) with a phenomonex C18 column (150 × 2.1 mm i.d. 1.8 µm particle size) and detected by MS/MS (AB Sciex API 3000) operating in MRM in negative mode with electrospray source. LC was run in a gradient at 300 µL min^{-1} with mobile phases of (A) water/methanol (95:5) and water/methanol (5:95) both with 5 mM ammonium formate and 0.1% formic acid. Gradient started at 95% A holding for 3 min, before linear increase to 60% B in 12 min and a gradient to 95% B at 15 min holding for 3 min before returning to initial conditions for 8 min. Determination of compounds was by MRM using paired ions and validation of compound used multiple daughter ions (Table 2).

Assessment of hazards posed to bees

A hazard assessment of dietary exposure of the adult honeybee foragers, and a worker nurse bee for the oral exposure from nectar and pollen consumption was ran, using a range of LD_{50}s from literature (Table 1). This is not an extensive literature review of heath risks but does provide some understanding of the ranges typically found. Foraging bees consume 13 mg of pollen and 400 mg of nectar that can be approximated to 80 mg of honey. A brood nurse bee consumes 6.6 mg of pollen and 40 mg of honey (Rortais et al. 2005). These two bees were used to identify hazard between two bee types. Risk was assessed by incorporating the frequency of detection of individual compounds based upon the dose (Eq. 1 (Sanchez-Bayo and Goka 2014))

$$\text{Risk} = \frac{\text{frequency}\,(\%) \times \text{residue dose}\,(\text{ng})}{\text{LD50}\,(\text{ng bee}^{-1})} \quad (1)$$

For each individual compound three tiers of risk were assessed; a lowest risk where the lowest measured dose is compare to the greatest LD_{50} measured in literature, an average risk using the mean residue dose and LD_{50} and a maximum risk incorporating the lowest LD_{50} with the greatest dose. All three tiers were used as to the authors knowledge no specific LD_{50} assessments of the Egyptian subspecies of Western honeybee to NIs has been performed therefore it is unknown their precise risk. In assessments, there are a wide range of cofactors, such as individual genetics, temperature, and how a toxic compound is applied to the organism that will cause effects so a wide scope of effects are assessed. Non-lethal behavioral of NIs were also considered for four compounds (acetamiprid, clothianidin, imidacloprid, thiamethoxam) (Decourtye et al. 2003, 2004a, b; Dively et al. 2015; Henry et al. 2012; Lu et al. 2014).

As there is documented synergism between ergosterol inhibiting compounds including the cyano-substituted NIs the risk may be considered cumulative thus the sum of

Table 2 Mean concentrations of detected NIs from both spring and summer sampling and their metabolites in honey, pollen and bees with minimum and maximum concentrations presented in brackets

Compound		Honey ng g^{-1}	Pollen	Bees	Bees ng bee^{-1}	LOQ ng mL^{-1}	Ions *m/z*
Acetamiprid		4.5 (1.69–9.4)	13.63 (2.43–22.06)	ND		0.1/0.5	**223.1**–126.0/99.0
	F (%)	19	6	3			
Clothianidin		ND	4.53	4.41 (0.06–10.38)	4.0×10^{-3} (5.4×10^{-5}–9.3×10^{-3})	0.4/0.5	**250.2**–169.0/131.9
	F (%)	0	3	12			
Cl-MNG							**131.9**–73.0/89.0
Cl-TMG							**205.0**–132.0/113.0
Cl-TZMU							**206.0**–132.0/120.0
Cl-TZNG							**236.0**–132.0/155.0
Imidacloprid		0.87 (0.46–1.68)	6.15 (5.27–7.03)	ND		0.6/2.0	**256.1**–209.0/175.1
	F (%)	8	6	0			
Olefin		0.94	ND	ND		0.5/0.0	**254.1**–205.2/171.1
	F (%)	3	0	0			
5-Hydroxy		0.58 (0.35–1.08)	11.17 (2.1–41.67)	7.62 (1.04–34.26)	6.9×10^{-3} (9.4×10^{-4}–3.1×10^{-2})	1.0/1.0	**272.1**–191.0/225.1
	F (%)	16	15	44			
Urea							**212.1**–128.1/78.0
Desnitro olefin		0.52	5.01 (1.90–9.04)	0.57		1.0/1.0	**209.8**–126/90.0
	F (%)	5	21	3			
Desnitro HCl		ND	0.26/3.15	16.38 (2.44–26.78)	1.5×10^{-2} (2.1×10^{-3}–2.4×10^{-2})	1.5	**211.1**–126.0
	F (%)	0	6	20			
6-Chloronicotinic acid		0.62	ND	ND		1.0/1.0	**126.0**–77.0/121.9
		3	0	0			
Thiamethoxam		18.84	12.35/15.50	1.90/5.64	1.7×10^{-3}/5.1×10^{-3}	2.1/1.0	**291.9**–181.0/211.0
	F (%)	3	6	8			
Dinotefuran		0.57 (0.31–0.96)	7.61 (1.26–17.45)	37.05 (0.38–74.31)	3.3×10^{-2} (3.4×10^{-4}–6.7×10^{-2})	0.8/2.0	**203.2**–129.1/157.1
	F (%)	38	24	76			
Urea		0.42 (0.23–0.66)	6.55	20.33 (0.27–41.83)	1.8×10^{-3} (2.4×10^{-4}–4.3×10^{-3})	1.0/1.0	**159.0**–102.0/67.0
	F (%)	14	3	20			
Phosphate		0.88 (0.73–1.03)	3.4 (1.57–5.99)	0.52	4.7×10^{-4}	2.0/2.0	**158.1**–57.0/109.1
	F (%)	22	18	4			
Nitenpyram							**271.0**–99.0/126.0
Thiacloprid							**253.1**–98.9/125.9

Compounds given in bolded text are parent compounds while those not are metabolites. Non-determined (ND) are noted and where $n = 1$ or $n = 2$ for a compound the value(s) are expressed while grey areas are for non-determined in any matrix. *F* is the frequency of detection in the matrix above given as a percentage (%). Bee concentration is estimated given a typical bee mass of 90 mg per bee. Limits of Quantification (LOQ) are for the MRM pairs, set as 10 times the baseline. Ions are the parent ion and the greatest intensity product ions

exposure can be assessed. Where a value greater than 1 implies a risk that the effect level has been exceeded. In the case of LC_{50}, this implies 50% mortality, and in non-lethal concentrations a deleterious observed effect.

Two further assessments of exposure were introduced the first is the fixed dose approach described by Sanchez-Bayo and Goka (2014). Where assuming that daily exposure rate is constant a time to reach LD_{50} for individual compounds

can be estimated using the following formula (Eq. 2).

$$\text{T50 (days)} = \frac{\text{LD50 (ng bee}^{-1}\text{)}}{\text{daily dose (ng)}} \quad (2)$$

A further assessment of the risk based upon the total lifespan of the bee was also used. The average lifespan of brood and forager also varies with brood bees living approximately 10 days and foragers 30 days in summer (Sanchez-Bayo and Goka 2014). No use of the overwinter lifespan for drones was used. The reason is that in temperate climates bee dormancy occurs for 90 days or more but is unclear if Egyptian bees behave similarly. A further assumption in this study is that dietary LC_{50}s remain stable, and residues are accumulated and not degraded or lost from the bee.

Quality control and assurance

QuEChERS extraction used in this study was validated previously with spike recoveries of NIs from honey or sodium sulphate at a range of concentration giving ~80% recovery of target compounds but recovery of surrogate standard of ~60% for actual matrices (Codling et al. 2016). In that study 2 samples exhibited effects of the matrix and had poor recoveries so a smaller final volume 4 mL rather than 6 mL of sample was used in this study. A similar extraction method was previously validated (Tanner and Czerwenka 2011) with 60–114% recovery of spiked honey.

Triplicate extraction blanks of honey heated to 80 °C for 8 h, baked sodium sulphate, water, sucrose solution and blank vials were run alongside samples to test for contamination. No field blanks were taken so there is some uncertainty of contamination. All blanks were <LOQ for parent compounds and for metabolites the greatest concentrations were seen in commercial cane sugar sucrose solution at <0.2 ng g^{-1} dm.

Concentrations of imidacloprid, acetamiprid, thiacloprid, thiamethoxam, flonicamid and nitenpyram were determined by use of a seven-point calibration curve (1–300 ng mL^{-1}). Concentrations of investigated metabolites were calculated by using parent calibration curves so results should be treated as semi-quantitative. For imidacloprid the metabolites were olefin, 5-hydroxy, urea, desnitro, desnitro –HCl and 6-Chloronicotinic acid. For clothianidin the metabolites, MNG, TMG, TZMU and TZNG were investigated (Kim et al. 2012). Dinotefuran and its metabolites (urea and n-phosphate) were calculated from acetamiprid calibration curve since this was the nearest molecular mass available. Quantification was based on multiple, paired parent and transition ions, 2–3 pairs per compound, with an LOD set as three times the baseline and LOQ at 10 times the baseline. Compound identification was limited to positive identification of all parent/daughter ions and having a calculated concentration of <10% variability. Mean recoveries of D^3-Imidacloprid were 79% ± 31 (median 69%) for bees (n = 25), 83% ± 49 (median 66%) for pollen (n = 36), and 77% ± 39 (median 71%) for honey (n = 35). Processed samples were not corrected for recoveries but values less than the method detection limit (MDL), which was set as the mean of the extraction blank plus 3 times the SD, were omitted.

Results and discussion

In total 25, 37 and 33 samples of bee workers, honey and pollen were processed, respectively. Of the 45 hives that were sampled, from some only limited material was collected, while some material was lost during transport and some vessels were compromised in storage. Of the 7seven parent compounds for which authentic reference standards were available, flonicamid, nitenpyram and thiacloprid were not detected in any sample. Dinotefuran was found in the most samples (n = 34, Table 2). However, lacking a reference standard for this compound concentrations are reported as estimates and is not discussed in detail, (SI Tables 1, 2, 3 contain the raw concentrations detected).

Of the metabolites of imidacloprid, (n = 6), clothianidin, (n = 5) and dinotefuran (n = 2), none of the clothianidin metabolites were detected in any sample above the LOD. Imidacloprid-urea was not detected in any sample above the LOQ, while the olefin and 6-chloronicotinic acid were determined in only one sample, (Table 2). Frequencies of detection of NIs were similar to those reported in a review of NIs in bees and bee products, (Blacquière et al. 2012).

Concentrations of NIs in bees were similar to those described in previous studies, (Bacandritsos et al. 2010; Calatayud-Vernich et al. 2016; Codling et al. 2016; Cutler and Scott-Dupree 2007; Hladik et al. 2016; Pistorius et al. 2009; Tanner and Czerwenka 2011). Concentrations of clothianidin in bees (mean 4.41 ng g^{-1} wet mass (wm) in this study) were consistent with those observed previously for dead/dying bees, (3.8–13.3 ng g^{-1}, wm) (Krupke et al. 2012). While concentrations measured in pollen in this study (4.41 ng g^{-1} wm) were between those concentrations for bees reported to be healthy and impacted in that same study, 2.9 and 10.7 ng g^{-1} wm, respectively (Krupke et al. 2012).

No effect levels (NOEL) for oral toxicity for acetamiprid, clothianidin and imidacloprid was estimated at 403, 0.95, and 1.5 ng bee^{-1}, respectively, (USEPA 2014). In no single sample of either pollen or honey was the NOEL threshold exceeded in this study and even combining the maximum concentration detected in both pollen and honey the NOEL values are still not exceeded.

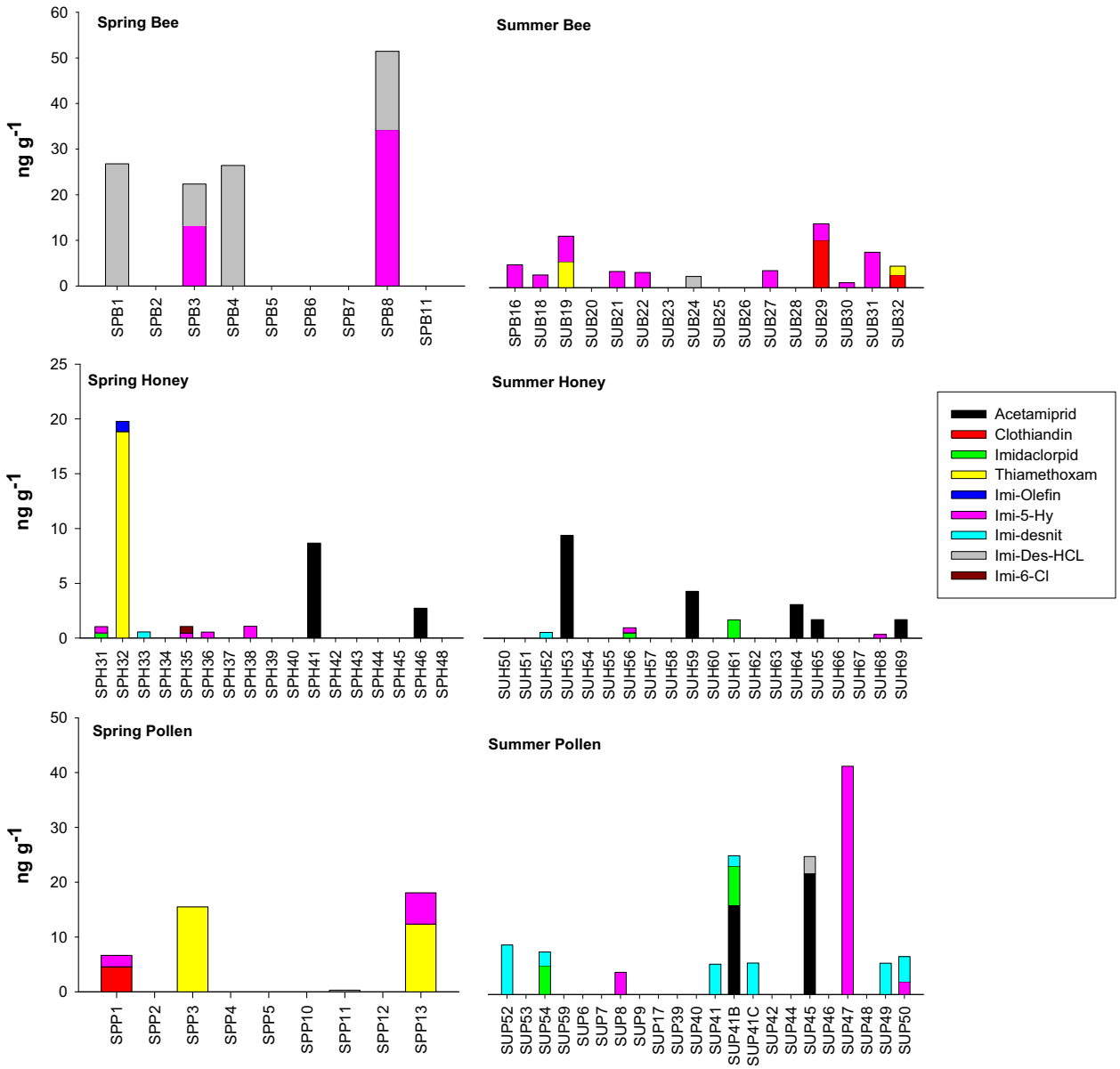

Fig. 2 Concentrations of NIs and their metabolites in spring (SP) and summer (SU) at various locations in the Nile River Delta of Egypt for bees (B), honey (H) and pollen (P)

Differences between spring and summer

Concentrations of NIs varied between spring and summer (Fig. 2). In the spring sampling none of the parent compounds were above the LOQ for bees in any sample while in the summer thiamethoxam ($n = 2$) and clothianidin ($n = 3$) were identified in a limited number of samples. As clothianidin is a breakdown product of thiamethoxam it is not possible to determine whether clothianidin contamination is a metabolite or from direct uptake. The metabolites of thiamethoxam and clothianidin were not detected in any bee samples above the limit of detection. The metabolites of imidacloprid, 5-hydroxy-imidacloprid and desnitro were measured in both spring and summer samples, with a greater concentration in the spring 19.7 and 17. ng g^{-1} for 5-hydroxy-imidacloprid and desnitro –HCl, respectively, in comparison to 2.4 and 3.9 ng g^{-1} wm during summer.

In honey parent compounds, acetamiprid and imidacloprid were observed in both spring and summer with no differences in concentrations between the two seasons. Metabolites of imidacloprid were detected at a similar frequency and at similar concentrations for example imidacloprid-5-Hydroxy was measured in four samples with a mean of 0.7 ng g^{-1} wm during spring and two samples at 0.4 ng g^{-1} wm during summer.

In pollen collected during spring clothianidin and thiamethoxam were the parent compounds detected, while during summer acetamiprid and imidacloprid were the only parent compounds observed. For the metabolites of imidacloprid, SU47 was an exception with imidacloprid-5-hydroxy at 41.7 ng g^{-1} wm. excluding this concentration and frequency of detection of 5-hydroxy and desnitro-HCl were similar between summer and winter while imidacloprid-desnitro was detected only during summer.

Given that imidacloprid and its metabolites were observed in honey and pollen and the metabolites were determined in bees it further links the dietary pathway. In addition, imidacloprid is metabolized rapidly in the bee (Suchail et al. 2004) and that the samples were transported long distances it is not surprizing that the parent molecules were not observed in the bee. Coupled to this is the fact that foliar spraying is not a typical application for most NIs diet would be the primary route of exposure.

Dietary risk to bees

For the three levels of risk calculated for the total exposure to NIs at a worst-case scenario taking the lowest reported LD_{50} and comparing to the greatest concentrations of residues there is a significant risk to the drone and nurse bees on a daily basis, (Table 3). However, over a lifetime of exposure all scenarios indicated significant risk to both nectar foragers and nurse bees. The assessment of non-lethal effects from oral exposure was also considered. In spring samples, the forager bee's behavior may be affected by a single daily oral dose, even at the lowest exposure risk to the mixture of NIs. While for nurse bees at the lowest risk scenario moderate risk is observed. Summer exposure to NIs on a daily basis is less of a risk to bee health than in spring mostly due to thiamethoxam being identified more often in spring.

Of all the compounds, observed thiamethoxam is the key compound driving the increased risk of mortalities contributing to 94% of the observed toxic effect in this study (SI Table 6 for risk based upon LC_{50} and SI Table 7 for sub-lethal oral exposure risk). Though the frequency of detection in honey and pollen was low just 3 and 6%, respectively, its effect on the risk was greatest. This observation is similar to that reported by Sanchez-Bayo and Goka (2014) in an assessment of residues in honey. Imidacloprid and the metabolites 5-hydroxy and desnitro pose a moderate risk in residues in a worst case of exposure and over the lifetime of exposure.

For most compounds the time required for individual compounds to reach levels of toxicity far exceed the lifetime of the individual bee. For acetamiprid, for example the average exposure time for a foraging bee to reach 50% mortality at the residue level observed is 45 years (SI Table 6). However, thiamethoxam would reach levels of 50% mortality on average within 7 days in foragers and 14 days for nurse bees. Both imidacloprid and clothianidin in a worst-case exposure scenario be a moderate risk to bee mortality. Looking at the non-lethal concentrations observed to cause behavioral effects from acetamiprid, clothianidin, imidacloprid and thiamethoxam, thiamethoxam in residues would potentially affect the behavior of foragers within 2 days of oral exposure and 4 days for nurse bees. For imidacloprid, residue concentrations in summer would reach levels within the lifetime of the bee that would have deleterious effects on 50% of the foragers.

Table 3 Risk of oral dose to NIs during spring summer and both seasons for mean (average case) and maximum (scenarios and lowest case scenarios (least exposure)

		TDI LD_{50} risk		TDI-NO(A)EL	
		Drone	**Nurse**	**Drone**	**Nurse**
Daily risk					
Mean	Sum	0.642	0.320	2.642	1.318
	Spring	0.626	0.312	2.344	1.169
	Summer	0.033	0.016	0.446	0.223
Min	Sum	0.246	0.123	1.611	0.804
	Spring	0.245	0.122	1.485	0.741
	Summer	0.006	0.003	0.199	0.099
Max	Sum	2.642	1.318	6.742	3.364
	Spring	2.204	1.100	5.844	2.916
	Summer	0.532	0.266	1.093	0.546
Lifetime exposure risk					
Mean	Sum	19.251	3.202	79.268	13.184
	Spring	18.784	3.124	70.324	11.694
	Summer	0.985	0.164	13.394	2.229
Min	Sum	7.388	1.229	48.318	8.036
	Spring	7.359	1.224	44.543	7.407
	Summer	0.189	0.031	5.975	0.995
Max	Sum	79.265	13.185	202.268	33.642
	Spring	66.123	10.996	175.330	29.158
	Summer	15.955	2.656	32.799	5.458

Least exposure uses the minimum measured dose so is a reflection of the lowest observable concentration. TDI-NO(A)EL is based upon the non-lethal oral exposure effects

Conclusions

Though the use of NIs is not well documented in Egypt, concentrations and frequency of detection indicate that use is similar to that of the US and European farms. Further study is needed with more frequent monitoring across the Nile Delta to assess regional and temporal trends of NIs to

more fully understand the impact that these compounds may have throughout the year.

Of all the NIs observed thiamethoxam poses, the most significant risk to the health of the bee and the concentration observed in residues is of concern. However, the frequency of positive identification is still low. The risks observed in this study may underestimate the effects of cumulative toxicity for mixtures and the effects of sub-lethal oral doses on behavior. It has also been observed that continuous exposure to NIs cause the LD_{50} concentration to decline over the exposure period (Rondeau et al. 2014). Therefore, the toxicity will increase over the lifetime of the bee (Suchail et al. 2001). There is also need to factor in contact effects of NIs, as acetamiprid and thiacloprid are used as foliar insecticides, however as other NIs are systemic the risks from contact events may be less.

For non-lethal effects, there appears to be a great risk to the bee from thiamethoxam and imidacloprid but further work is needed on long-term assessments. Effects like immune suppression (Di Prisco et al. 2013) associative learning reduction and foraging behavior have all been observed (Blacquière et al. 2012; Sánchez-Bayo et al. 2016) in previous studies and models but implementing these diverse factors into a risk assessment as a single dose response may need more thorough assessment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval The bees used in this study were handled under the ethical guidelines of Tanta University Egypt, and all handling of hives was performed under the guidance and supervision of professional apiarists.

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