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Original article

Potential impacts of synergism in honeybees (*Apis mellifera*) of exposure to neonicotinoids and sprayed fungicides in crops

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Abstract – Assessment of the toxicity of individual pesticides to honeybees is routinely assessed. However, few data have been generated for realistic mixtures of neonicotinoid insecticides and fungicides particularly with regard to exposure levels used. Assessment of the effects of exposure of bees to predicted residues following sprayed applications of ergosterol biosynthesis inhibitor fungicides on the contact and oral toxicity of a range of neonicotinoid insecticides (thiamethoxam, clothianidin, imidacloprid and thiacloprid) showed only low levels of synergism (<3-fold maximum). Further studies showed that the scale of increase in toxicity was fungicide dose dependent with greater synergy of oral toxicity of thiamethoxam following contact dosing with propiconazole. This underlines the need for the use of realistic exposure levels and routes in studies.

honeybees / synergism / EBI fungicides / neonicotinoids

1. INTRODUCTION

The regulatory risk assessment for pesticides in Europe includes assessment of the toxicity of both pesticide active ingredients and formulations to honeybees (*Apis mellifera* L.) (EFSA 2013). Pesticide formulations often include multiple active ingredients; thus, any differences from the predicted toxicity of component actives can be readily established. However, the risks associated with the use of sequential pesticide sprays or tank mixes on crops (with a few exceptions, e.g. organophosphorus pesticides) are not routinely assessed. There is evidence in the literature that exposure to multiple pesticides may result in synergistic effects (i.e. toxicity is more than additive), and these can be predicted based on mode of action. A clear example is the increased toxicity of pyrethroid insecticides to honeybees in the presence of ergosterol biosynthesis inhibitor (EBI) fungicides, which inhibit the microsomal monooxygenases (P450s) responsible for oxidative detoxication (Brattsen et al. 1994; Hagler et al. 1989; Pilling 1992; Colin and Belzunces 1992; Pilling et al. 1995; Johnson et al. 2012). However, the potential for exposure to multiple pesticides is not limited to combinations of sprayed products. Systemic seed treatments are increasing in popularity as their use can result in both lower numbers of pesticide spray applications and improved control of pests/diseases at

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growing shoots. However, transport of systemic active substances within the plant may also lead to residues in pollen, nectar and guttation fluid resulting in the potential for exposure of pollinators to mixtures when other pesticides are applied as sprays. The systemic pesticides of particular current interest are the neonicotinoid insecticides. These have been reported to act synergistically with compounds which inhibit the P450s involved in their metabolism; exposure to triazole fungicides has been shown to increase the toxicity of some neonicotinoids several hundred fold (e.g. thiacloprid with propiconazole) (Iwasa et al. 2004). This suggests that even at the very low (μg/kg) levels of neonicotinoid insecticides detected in pollen and nectar (Blacquière et al. 2012), toxic effects may occur with co-exposure to fungicides. However, as is the case with many studies (see Thompson 1996), the effects of realistic exposure levels are rarely investigated. The approach used in this study was to assess the toxicity of imidacloprid, thiamethoxam, clothianidin and thiacloprid alone and in combination with realistic exposure rates of fungicides. The fungicides (flusilazole, propiconazole, tebuconazole and myclobutanil) were identified as those used in the UK on flowering crops, which may also have been grown from treated seed and thus contain residues of the neonicotinoids in pollen, nectar or guttation fluid.

2. MATERIALS AND METHODS

All insecticides and fungicides were obtained from Sigma-Aldrich as Pestanal analytical standards (purity: thiamethoxam 99.7 %, clothianidin 99.9 %, imidacloprid 99.9 %, thiacloprid 99.9 %, flusilazole 99.8 %, propiconazole 98.5 %, myclobutanil 99.4 %, and tebuconazole 99.5 %). All contact test dilutions were prepared in acetone (Analar) and oral dilutions in 50 % *w*/*v* aqueous sucrose (in deionised water) with pre-dilution in acetone as required (no oral test solution contained greater than 1 % acetone). Different concentrations of the same substance were applied in the order of rising concentrations. All test solutions were freshly prepared and then stored at 4–10 °C for up to 2 h until required for dosing.

Worker honeybees (*A. mellifera* L.) were sourced from National Bee Unit, Fera. The adult worker bees were collected from colonies which had low levels of adult bee diseases (free from acarine and amoeba) and which had not been treated with varroacides during the previous 4 weeks.

The realistic worst-case exposure of honeybees to fungicides immediately after a spray application to crops was calculated from the maximum approved application rate for the fungicides in UK crops and highest mean residues (contact) on honeybees after application of 20 g tracer/ha (Koch and Weisser 1997) (35.77 ng/bee). The realistic worst-case exposure doses per bee used were 0.358-μg flusilazole/bee, 0.161-μg myclobutanil/bee, 0.224-μg propiconazole/bee, and 0.447-μg tebuconazole/bee. Pilot studies undertaken with these fungicides (including higher doses of propiconazole) showed no overt toxicity.

The toxicities of imidacloprid, thiamethoxam, clothianidin, and thiacloprid alone and in combination with the fungicides were determined in standard OECD honeybee contact and oral toxicity tests (OECD 1998a, b). Using two routes of exposure allowed assessment of the toxicity by ingestion (pollen or nectar) and by contact with treated surfaces (e.g. dried guttation residues). Fungicides and insecticides were dissolved in acetone for contact dosing and diluted in 50 % *w*/*v* sucrose for oral dosing. There were at least five dose rates for the insecticide (with a maximum of 2-fold between doses) and three replicates per dose.

Worker bees were collected from the hive by using a small amount of smoke, gently shaking them from the combs and transferring them into cylindrical mesh cages. In the laboratory, the mesh cages were placed into the incubator (25±2 °C, 65±5 % relative humidity) until needed for the test. Immediately prior to treatment, each group of bees in its mesh cage was anaesthetised by placing the cage into a 2-L beaker filled with carbon dioxide gas for a maximum of 2 min. Any bees which were visibly damaged were excluded from the study.

The test unit used to assess bee mortality consisted of a 9-cm inverted triple-vented plastic petri dish within which a 3-cm plastic petri dish sucrose feeder

was attached. A filter paper liner was placed in each test unit. For oral dosing, a small glass feeder was placed inside the test unit and a hole inserted in the lid of the test unit to allow its removal at the end of the exposure period. The test units were placed at 25 ±2 °C and 65±5 % relative humidity in the dark except during observations.

To ensure that variations were not due to differences in timing of the studies in the season, each insecticide was assessed with each of the fungicides in parallel with tests on the toxicity of the insecticide alone. The studies generated mortality data for the systemic insecticide with at least five doses in combination with the residues predicted from the maximum estimated exposure for the fungicide (the maximum dose likely to occur) to determine the median lethal dose (LD_{50}) for each combination.

For contact dosing, the bees were anaesthetised with carbon dioxide immediately before dosing and gently tipped out onto filter paper, and the workers were counted into the petri dish. Each bee was dosed on the dorsal thorax with a 1-μL drop of the insecticide and/or 1-μL drop of the fungicide concentration or two 1-μL drops of acetone (for controls) using a micropipette. The lid was placed on the cage; the bees allowed to recover and kept in the incubator with a continuous supply of 50 % *w*/*v* aqueous sucrose solution as food.

For oral dosing, the bees were starved for 1.5–2 h before dosing. They were then anaesthetised with carbon dioxide immediately before dosing and gently tipped out onto filter paper, and the workers were counted into the petri dish cage. Each group of ten bees was offered 200 μL of a given concentration of the insecticide and/or fungicide (or controls as above), the dose being measured into a small, preweighed, glass feeder within the cage using a variable volume pipette. After 4 h (±30 min), the glass feeders were removed and weighed, and the sucrose feeders were filled with approximately 3 mL 50 % *w*/*v* aqueous sucrose so that bees had continuous access to sucrose for the remainder of the study. The dose consumed was determined by comparison of the weight of the dose remaining in the glass feeders with the weight of a known volume of the test solutions.

To assess the effects of the fungicide dose rate (exposure level) on the contact and oral toxicity of thiamethoxam, the effect of varying the contact doses of propiconazole was tested. In this test, all bees were dosed with propiconazole at 0, 0.0224, 0.224, 2.24 and 22.4 μg/bee and then to the contact or oral dose of thiamethoxam as above.

Observations of mortality and behaviour (knockdown or stumbling) in the contact dosing test were recorded 1 (±15 min), 4 (±15 min), 24 and 48 h (±30 min) after dosing. In the oral dosing test, the observations of mortality and behaviour (knockdown or stumbling) were recorded 1 (±15 min) and 4 h (±15 min) after dosing and 24 and 48 h (±30 min) after removal of the test feeders. The tests were established such that if significantly increased mortality was observed between 24 and 48 h, the observation period would be extended up to a maximum of 96 h for the test item treated and control groups.

The endpoint used to assess the effect of the mixtures on toxicity was the mortality rate after 48 h. This was appropriate as there was no significant increase in mortality (or abnormal behaviour) in the test units between 48 and 72 h (or 96 h where test units were re-checked for any indications of delayed mortality). Mortality was expressed as a dose-response relationship with an LD_{50} and 95 % confidence limits using probit.

3. RESULTS

3.1. Single actives

The data (Tables I and II) show that for thiamethoxam, clothianidin and thiacloprid, the acute contact and oral toxicity were similar to those previously reported (Agritox http://www.agritox.anses.fr/guides/guide-agritox-anglais.html). Stumbling and/or knockdown was observed at 4 h in almost all clothianidin-, thiamethoxam- and imidacloprid-treated cages (the doses were selected to assess the mortality rather than the behavioural effects), and the data were thus not suitable for the analysis of the dose-response approach required for assessing increased sublethal toxicity. None of the fungicides resulted in any toxic effects when they were tested at the doses used in the study.

3.2. Effects of fungicides on the toxicity of insecticides

Table I (and Figure S1) summarises the effects of co-exposure to contact doses of the insecticides with the EBI fungicides. The toxicity of thiacloprid and imidacloprid was virtually unchanged by co-exposure to the fungicides whereas thiamethoxam and clothianidin showed some indications of increased toxicity, although this was less than 3-fold. The highest level of synergy, 2.6-fold, was observed between tebuconazole and thiamethoxam. There was also no effect on the doses at which sublethal (stumbling/knock-down) effects were observed at 4 h in the thiacloprid-treated bees (in the case of imidacloprid, this could not be ascertained as sublethal effects were observed at all doses). Across all the fungicides, the data suggest that there was a relationship between the fungicide dose used and the observed increase in clothianidin and thiamethoxam toxicity.

3.3. Oral toxicity of combinations

Table II (and Figure S2) summarises the toxicity data generated following the oral exposure to both the insecticide and fungicide. Again, no increase in toxicity was observed when imidacloprid or thiacloprid doses were ingested in the presence of the fungicides. The highest level of synergy observed was less than 2-fold between clothianidin and tebuconazole. There was evidence of a relationship between the fungicide dose used and the toxicity of clothianidin but not thiamethoxam.

3.4. Dose-response relationships

The effects of varying the contact dose of the fungicide propiconazole on the contact and oral toxicity of the neonicotinoid thiamethoxam are shown in Table III. This shows the dose dependency of the change in both the contact and oral toxicity of thiamethoxam with the dose of propiconazole. Increasing the contact propiconazole, contact thiamethoxam dose ratio from 0.6:1 to 600:1 resulted in a 1.3- to 3.6-fold increase in toxicity of thiamethoxam. Increasing the contact propiconazole, oral thiamethoxam dose ratio from 0.35:1 to 350:1 resulted in a 2.4- to 8.3-fold increase in toxicity of thiamethoxam.

4. DISCUSSION

The effects of co-exposure to the EBI fungicides on the toxicity of neonicotinoids to honeybees vary between the active ingredients. Co-exposure to the EBI fungicides did not increase the contact or oral toxicity of imidacloprid. These data are in agreement with those of Iwasa et al. (2004) who showed that high doses (10 μg/bee) of piperonyl butoxide increased the toxicity of imidacloprid only 1.7-fold, and the data support the assertion that P450s are not key to the metabolism of imidacloprid.

In only two cases did a field realistic dose of EBI fungicide significantly increase the toxicity of the neonicotinoid insecticides (confidence limits of the LD_{50} estimate for the mixture did not overlap with those of the insecticides alone). These were contact doses of thiamethoxam with tebuconazole and oral doses of clothianidin with tebuconazole (Tables I and II). In both cases, the increase in toxicity was less than 3-fold, and in no case was synergy was observed above this level. This confirms the evaluation of the scale of synergism following realistic exposures identified by a number of authors (Deneer 2000; Laetz et al. 2009; Verbruggen and Van der Brink 2010).

This study has shown that the dose of fungicide that the bee receives is a key factor in determining the toxicity of the neonicotinoids. It explains the differences in synergy identified in this study when compared with published studies in which high levels of fungicides were used. Higher levels of synergy were identified at the maximum contact doses of propiconazole (22.4 μg/bee) used, where the sensitivity to an oral dose of thiamethoxam increased over 8-fold and sensitivity to a contact dose of thiamethoxam increased

Table I. Contact toxicity of combinations.

Insecticide	LD_{50} (μg/bee) (95 % CL)	+Myclobutanil (0.161 μg/bee)		+Propiconazole (0.224 μg/bee)		+Flusilazole (0.358 μg/bee)		+Tebuconazole (0.447 μg/bee)	
		LD_{50} (μg/bee) (95 % CL)	Synergism ratio	LD_{50} (μg/bee) (95 % CL)	Synergism ratio	LD_{50} (μg/bee) (95 % CL)	Synergism ratio	LD_{50} (μg/bee) (95 % CL)	Synergism ratio
Clothianidin	0.0350 (0.0155–0.0607)	0.0451 0.0363–0.0559	**0.78**	0.0312 0.0239–0.0393	**1.12**	0.0295 0.0230–0.0367	**1.19**	0.00287 0.0213–0.0368	**1.22**
Imidacloprid	0.0671 (0.0438–0.1018)	0.0409 0.0205–0.0663	**1.64**	0.0585 0.0379–0.0867	**1.15**	0.0475 0.0187–0.0912	**1.41**	0.0347 0.0161–0.0568	**1.93**
Thiacloprid	122.4 (90.56–238.9)	635.8 $184.1–3.51\times10^{7}$	**0.19**	434.9 156.8–65909	**0.28**	439.3 157.3–71052.1	**0.28**	266.8 128.9–3738.9	**0.46**
Thiamethoxam	0.124 (0.0768–0.3280)	0.0979 0.0804–0.1223	**1.27**	0.0638 0.0521–0.0788	**1.94**	0.0538 0.0254–0.1203	**2.30**	0.0479 0.0302–0.0757	**2.59**[a]

[a] Confidence limits do not overlap with those of insecticide alone

LD_{50} median lethal dose, *CL* confidence limit, *SR* Synergism ratio = insecticide LD50/(insecticide + fungicide) LD50

Table II. Oral toxicity of combinations.

Insecticide	LD_{50} (μg/bee) (95 % CL)	+Myclobutanil (0.161 μg/bee)		+Propiconazole (0.224 μg/bee)		+Flusilazole (0.358 μg/bee)		+Tebuconazole (0.447 μg/bee)	
		LD_{50} (μg/bee) (95 % CL)	Synergism ratio	LD_{50} (μg/bee) (95 % CL)	Synergism ratio	LD_{50} (μg/bee) (95 % CL)	Synergism ratio	LD_{50} (μg/bee) (95 % CL)	Synergism ratio
Clothianidin	0.00739 (0.00607–0.00903)	0.00597 0.00493–0.00732	**1.24**	0.00572 0.00467–0.00710	**1.29**	0.00441 0.00267–0.00762	**1.68**	0.00389 0.00305–0.00489	**1.90**[a]
Imidacloprid	0.536 (0.339–1.184)	1.075 0.5667–4.426	**0.50**	1.501 0.6972–14.44	**0.36**	1.180 0.6094–5.878	**0.45**	0.893 0.438–14.50	**0.59**
Thiacloprid	22.59 (16.39–37.42)	25.67 19.01–40.70	**0.88**	47.01 27.45–166.1	**0.48**	54.65 25.88–852.8	**0.41**	36.19 20.99–134.1	**0.62**
Thiamethoxam	0.0112 (0.00915–0.0135)	0.00742 0.00448–0.01123	**1.51**	0.00830 –	**1.35**	0.0103 0.00801–0.0136	**1.09**	0.00852 0.00688–0.01037	**1.31**

CL could not be estimated

LD_{50} median lethal dose, *CL* confidence limit, *SR* Synergism ratio = insecticide LD50/(insecticide + fungicide) LD50

[a] Confidence limits do not overlap with those of insecticide alone

Table III. Comparison of the ratio of propiconazole to the doses of thiamethoxam and the resultant LD_{50} (95 % confidence limits) in the contact and oral studies.

Contact dose propiconazole μg/bee	Ratio fungicide: thiamethoxam contact LD_{50}	Contact LD_{50} thiamethoxam μg/bee	SR	Ratio fungicide: thiamethoxam oral LD_{50}	Oral LD_{50} thiamethoxam μg/bee	SR
0	–	0.0373 (0.0297–0.0466)	–	–	0.0641 (0.0322–0.417)	–
0.0224	0.6	0.0288 (0.0132–0.0558)	1.3	0.349	0.0268 (0.0214–0.0395)	2.4
0.224	6	0.0247 (0.0182–0.0325)	1.5	3.49	0.0277 (0.0203–0.0477)	2.3
2.24	60	0.0134 (0.0109–0.0162)	2.8[a]	34.9	0.0265 (0.0195–0.0442)	2.4
22.4	600	0.0104 (0.00494–0.0144)	3.6[a]	349	0.00776 (0.00438–0.0177)	8.3[a]

[a] Confidence limits do not overlap with insecticide alone

LD_{50} median lethal dose, *SR* synergism ratio = insecticide LD50/(insecticide + fungicide) LD50

by 3.6-fold. It is interesting to note an apparent threshold effect in the oral dosing studies in that a 2-fold increase in activity was identifiable at the lower contact propiconazole dose rates (0.0224–2.24 μg/bee) with an 8.3-fold increase in toxicity only at the highest contact dose of propiconazole (22.4 μg/bee). The scale of the effect of propiconazole on thiamethoxam toxicity is similar to that described by Pilling and Jepson (1993) for lambda cyhalothrin. With a synergistic ratio of 16.6:1, propiconazole decreased the contact LD_{50} of lambda cyhalothrin 16-fold from 68 to 4.2 ng/bee in co-applied mixtures at field realistic ratios.

Contrary to the published data, in this study no synergy was observed with thiacloprid co-applied with the EBI fungicides. This is likely to be due to the lower (more field realistic) fungicide doses used in this study. Iwasa et al. (2004) used a 10-μg/bee contact dose of propiconazole 1 h before exposure to neonicotinoids and identified a 559-fold increase in the toxicity of thiacloprid. Schmuck et al. (2003) showed an increase in mortality from 3 to 70 % when they co-applied thiacloprid with 3-μg/bee contact dose of tebuconazole.

Where synergy was observed there were differences in the effects shown between the two routes of exposure. Midgut metabolism is important in the detoxification of chemicals in the honeybee (Gilbert and Wilkinson 1974); simply cutting the midgut longitudinally resulted in 90 % reduction in aldrin epoxidase activity, and the midgut is known to contain monooxygenase inhibitors (Gilbert and Wilkinson 1975). This suggests that oral exposure of honeybee to neonicotinoids is likely to result in metabolism of the xenobiotic within the gut whereas contact exposure to both chemicals requires both transfer of the pesticide through the cuticle and metabolism within the body of the insect. The health of the midgut is therefore key in the detoxification following oral exposure. Therefore, the sensitivity to pesticides by ingestion may differ if disease or other pesticides are present. This may provide some explanation for the oral toxicity of imidacloprid in this study being 2 orders of magnitude lower than the published value while the contact LD_{50} was similar to published values. Previous data published by Fera (Defra 2007) is consistent with this and the susceptibility of bees to imidacloprid is reported to be wide-ranging (Schmuck et al., 2001, Suchail et al., 2001, Nauen et al., 2001) although there is no known mechanism. Factors may include feeding honey from

emergence rather than sucrose alone as prophylactic induction of P450s may occur through consumption of pollen and honey flavonoids enhancing bee survival (Johnson 2008). Understanding the impact of mixtures on toxicity following oral exposure is important despite the paucity of studies on this more realistic route of exposure where residues are present in pollen and nectar.

In assessing the impact of the data, it should be remembered that reported levels of neonicotinoid seed treatments in nectar and pollen are usually <20 μg/kg (Blacquiere et al. 2012; EFSA 2013), and therefore, exposure to an acutely toxic dose even at the maximum synergy observed is unlikely. Foraging bees are exposed via ingestion of contaminated nectar but consume virtually no pollen, and pollen intake is low in other adult bees within the hive (Rortais et al. 2005). Further work is required to understand the effects of synergists on non-lethal endpoints (Desneux et al. 2007). There are no published data on the relative impacts of synergists on sublethal and lethal endpoints, but it is important to understand whether endpoints such as foraging efficiency (Henry et al. 2012, Mommaerts et al. 2011) are also affected.

Honeybees contain a far more limited number of P450s than in other insects (Johnson 2008). In the absence of metabolism information specific for thiamethoxam in honeybees, there are several possible explanations of the increased toxicity in the presence of propiconazole observed in honeybees:

- An increased rate of conversion to clothianidin by induction of the P450s catalysing this conversion, or that of the *N*-desmethylation of thiamethoxam, the former is also a potent acetylcholine agonist—there are very few examples of induction of P450s in bees by xenobiotics although it has been demonstrated by quercetins in honey (Johnson 2008).
- Inhibition of the conversion to either clothianidin and/or *N*-desmethylthiamethoxam, which increases the availability of the parent compound for conversion to the alternative metabolite or to act as an agonist in its own right.
- Inhibition of metabolism of either desmethylthiamethoxam and/or clothianidin increasing the levels of these metabolites

All published studies to date have reported effects of combined acute exposure to pesticides, but oral exposure to systemic pesticide residues in pollen and nectar is likely to be chronic. Although the residue in nectar from a spray application of fungicide can be estimated (EFSA 2013), assessing the effects of chronic exposure should also take into account the exposure pattern and the rate of metabolism as these are both likely to impact on observed effects of a synergist. The interaction of these factors affecting exposure makes the design of a suitable laboratory assay challenging, e.g. is continuous or pulsed exposure (Defra 2009) more realistic for a foraging bee?

These results show that while there was some evidence of synergism between some of the fungicides and insecticides tested at realistic levels of exposure, this was at a relatively low level with usually no more than a 3-fold increase in toxicity. While higher levels were detected for propiconazole and thiamethoxam (up to an 8.3-fold increase), the conditions under which sufficient exposure to the fungicide would occur, although theoretically possible, they may only occur in a small number of such combinations of fungicide and insecticide exposures.

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Impacts potentiels sur les abeilles (*Apis mellifera*), de la synergie entre les néonicotinoïdes et les fongicides pulvérisés dans les cultures

Abeille / traitement phytosanitaire / culture de plein champ / niveau d'exposition / toxicité

Mögliche synergistische Effekte von Neonikotinoiden und fungiziden Spritzmitteln auf Honigbienen (*Apis mellifera*)

Honigbienen / synergismus / EBI fungizide / neonikotinoide

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