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# Residues of Pesticides in Honeybee (*Apis mellifera carnica*) Bee Bread and in Pollen Loads from Treated Apple Orchards

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**Abstract** Honey bee (*Apis mellifera carnica*) colonies were placed in two apple orchards treated with the insecticides diazinon and thiacloprid and the fungicide difenoconazole in accordance with a Protection Treatment Plan in the spring of 2007. Pollen and bee bread were collected from combs inside the hives. The residue of diazinon in pollen loads 10 days after orchard treatment was 0.09 mg/kg, and the same amount of residue was found in bee bread 16 days after treatment. In pollen loads 6 days after application 0.03 mg/kg of thiacloprid residues and 0.01 mg/kg of difenoconazole were found on the first day after application. Possible sub-lethal effects on individual honey bees and brood are discussed.

**Keywords** Insecticide · Fungicide · Analyses · Diazinon · Thiacloprid · Difenoconazole

Honey bees (*Apis mellifera* L.) collect nectar and pollen from flowers for their food and for brood development, so are useful for collecting material from the environment, and have been used to assess atmospheric and other types of pollution (Kevan 1999). Among the various routes of pesticide exposure for bees and other pollinating insects, pollen is particularly relevant, as the whole bee colony can potentially be exposed (Russel et al. 1998; Thompson and Hunt 1999). The safest time to apply pesticides in the field is thought to be during the night and early morning before bees are foraging. Another source of risk to bees is from treating a non-flowering crop when nearby cover crops, weeds and wildflowers are in bloom. These may be attractive nectar sources and may become contaminated by spray drift. A method for assessing the risk for honey bees from pesticide exposure via pollen was developed by Villa et al. (2000). Hazards of insecticides to honey bees have been reported in many studies (e.g. Koch and Weisser 1997; Villa et al. 2000; Waller et al. 1984; Schmuck et al. 2001; Bonmatin et al. 2003). Pollen loads have been analysed for pesticide residues to monitor weakness of honey bee colonies (Porrini et al. 2003; Chauzat et al. 2006). Two organophosphate compounds, coumaphos and diazinon, were examined for effects of sublethal exposure on odour learning and generalization in honey bees by Weick and Thorn (2002) and effects of pesticides on honey bees and brood have been studied by McKenzie and Winston 1989; Papaefthimiou et al. 2002; Rosiak 2002; Gregorc et al. 2004; Silva-Zacarin et al. 2006.

"Oleodiazinon" is a mixture of paraffin oil and diazinon, an organophosphorus insecticide and acaricide. It is used throughout the world to control a wide range of insects and mites on a range of crops and is toxic to honey bees. "Calypso" is an insecticide using thiacloprid as the active ingredient and is chemically related to imidacloprid, which has an effect on the hypopharingeal glands of worker honey bees (*Smodiš Škerl and Gregorc, submitted for publication*). Difenoconazole is used against pests on the apple and pear trees. In our survey, we made an attempt to detect possible residues of thiacloprid and difenoconazole in pollen loads and bee bread. We therefore performed an investigation in order to assess the level of residues in pollen collected by bees after application of the most commonly used pesticides in orchard areas; diazinon, thiacloprid, difenoconazole. We aimed to determine whether properly applied pesticide treatments in the field can

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be detected in pollen collected by honey bees using a pollen trap or bee bread stored in the comb. Pollen is used for feeding brood, therefore could have an adverse effect on brood and colony development. This is the first attempt to monitor the level of pesticides in pollen collected by honey bees in an orchard area in Slovenia.

## Materials and Methods

Experimental apiaries of honey bee (*Apis mellifera carnica* Pollm.) colonies were established in three sites: an apple orchard at Brdo near Lukovica, in the Ljubljana region; in an orchard at Čadovlje and a control apiary in Senično, both in the Gorenjska region. Two honey bee hives at each site were fitted with pollen traps for pollen collection. Colonies were visited five times in spring 2007 (April–May) before and after pesticide applications by the fruit growers. The colonies in Senično were used as controls in an area untreated with pesticides. Pollen loads of approximately 50 g were collected from the pollen traps into plastic cups after the field application of two insecticides: "Oleodiazinon" (a.i. diazinon) at a rate of 15 L/ha; and "Calypso SC 480" (a.i. thiacloprid) at a rate of 0,2 L/ha; and the fungicide "Score 250 EC" (a.i. difenoconazole) at a rate of 0,2 L/ha. The use of these substances was legally authorized in Slovenia in 2002 ("Oleodiazinon"), 2004 ("Calypso SC 480") and in 1999 ("Score 250 EC"). Diazinon is toxic to honey bees whilst thiacloprid and difenoconazole are thought to be harmless. The pesticides used were chosen because they are frequently used in orchards and are toxic to honey bees. The fruit growers sprayed the pesticides at night or early in the morning when the honey bees were inside their hives. Grass in the orchards was regularly cut down. Samples of pollen were collected one, 6 and 10 days after the field treatments from colonies located at both of the treated sites and from the control colonies. Samples of bee bread were also collected from comb 16 days after the pesticide application. The samples were immediately frozen, transported and stored in the laboratory at −20°C until analysed.

Analyses were performed in the Central Laboratory of the Agricultural Institute of Slovenia. Residues of the three possible contaminants were sought through individual or multiresidue analysis. Pollen samples from the two hives at each site were pooled, and analysed as one sample per apiary. For the analyses 10 g of sample were taken.

Specific analyses to detect diazinon, thiacloprid and difenoconazol were carried out. In the laboratory, the pollen samples were centrifuged for 4 min with 50 mL acetone, petroleum ether and dichloromethane in a ratio of 1:2:2. The liquid phase was decanted, evaporated to approximately 2 mL and dried with nitrogen flow (Makovi et al. 1999). The residue was dissolved in 8 mL of a mixture of cyclohexane and ethyl acetate in a ratio of 1:1 and cleaned on a gel permeation chromatograph (Thier et al. 1987; 1992). After evaporation, 1 mL of the mixture of ethyl acetate and cyclohexane in a ratio of 1:1 (for GC/MS analysis) or 1 mL of methanol (for LC/MS/MS) was added. Quantification for thiacloprid and difenoconazole was conducted using liquid chromatography-tandem mass spectrometry (LC/MS/MS) (Bossi et al. 2002), with limit of detection (LOD) at 0.01 mg/kg. Diazinon was quantified using gas chromatography mass spectrometry (GC/MS) (Fillion et al. 2000), LOD was 0.02 mg/kg. Fortified sample recoveries were 102.3% for diazinon, 100.0% for thiacloprid and 90.4% for difenoconazole.

## Results and Discussion

No dead honey bees were observed in front of the hives after treatment. The results of chemical analysis of pollen loads and bee bread are shown in Table 1. Diazinon and thiacloprid were detected in pollen loads on the first day after the pesticide application, but decreased after 6 and 10 days. Three days after treatment, diazinon residues in bee bread were less than the level of detection, but were detected 16 days after application at Brdo and 18 days after application at Čadovlje. At Brdo, thiacloprid and difenoconazole were detected one day after application, but thiacloprid was not detectable after 6 days. At Čadovlje, thiacloprid was found in pollen loads 6 days after application, but was not detectable in bee bread on day 10. No pesticide residues were detected in any pollen or bee bread samples collected from the control colonies.

Maximal exposure to systemic insecticides is to be expected among honey bees that consume the greatest amounts of contaminated pollen. Pesticides applied in the field are detectable in both pollen loads and in bee bread from inside hives. Both forms of pollen represent the protein sources for adult and the developmental stages of bees, large amounts being consumed by nurse bees, and to a lesser extent by larvae (Rortais et al. 2005). It is evident, therefore that honey bees can be exposed to lethal or sublethal doses of pesticides in their food, which can consequently have an impact on their development or longevity. The oral $LD_{50}$ for adult honey bees of diazinon is 0.09 μg, $LD_{50}$ of thiacloprid is 17.32 μg (Footprint 2007). The toxicological properties of insecticides and fungicides are indicative of adverse effects on adult bees and also sublethal effects at the tissue level causing morphological, histochemical and cytochemical changes. For example, imidacloprid or coumaphos treatment induced reduction the size of hypopharyngeal gland acinus and heat shock protein localisation in cell nuclei and cytoplasm. Coumaphos triggered an

**Table 1** Pesticide residues in pollen loads and bee bread from the colony brood chamber

| Sample | Sampling time | Area | Time after application (days) | Diazinon GC-MS (mg/kg) | Thiacloprid LC-MS/MS (mg/kg) | Difenoconazole LC-MS/MS (mg/kg) |
|---|---|---|---|---|---|---|
| Pollen loads | 3 April | Brdo | 1 | 1.98 | | |
| Bee bread | 6 April | Brdo | 3 | <LOD* | | |
| Pollen loads | 6–9 April | Brdo | 6 | 0.14 | | |
| Pollen loads | 12 April | Brdo | 10 | 0.03 | | |
| Bee bread | 18 April | Brdo | 16 | 0.09 | | |
| Pollen loads | 3 May | Brdo | 1 | | 0.09 | 0.01 |
| Pollen loads | 9 May | Brdo | 6 | | <LOD** | |
| Pollen loads | 6 April | Čadovlje | 6 | 0.18 | | |
| Pollen loads | 11 April | Čadovlje | 10 | 0.09 | | |
| Bee bread | 16 April | Čadovlje | 18 | 0.05 | | |
| Pollen loads | 4 May | Čadovlje | 6 | | 0.03 | <LOD** |
| Bee bread | 8 May | Čadovlje | 10 | | <LOD** | |
| Pollen loads | 6 April | Senično | / | | <LOD** | <LOD** |
| Pollen loads | 11 April | Senično | / | <LOD* | | |
| Pollen loads | 14 April | Senično | / | | <LOD** | |
| Pollen loads | 3 May | Senično | / | <LOD* | | |

<LOD* = Under level of detection 0.02 mg/kg; <LOD** = Under level of detection 0.01 mg/kg; GC-MS = Gas chromatography-mass spectrometry; LC-MS/MS = Liquid chromatography-tandem mass spectrometry

increased level of programmed cell death and imidacloprid extended necrosis (*Smodiš Škerl and Gregorc, submitted for publication*). In honey bee larvae exposed to Amitraz, both necrotic and apoptotic cell death have been demonstrated (Gregorc and Bowen 2000) indicative of subclinical effects and increased mortality as indicated in toxicological experiments conducted on bee brood and adult bees (Gregorc and Smodiš škerl 2007). Immunohistochemical methods can be useful for studying in situ tissue pathology, and indicate possibilities for monitoring the effects of infections and chemical environmental stressors on cell death in honey bee larval tissue.

The $LD_{50}$ for the three pesticides monitored in our survey was not achieved for individual bees as the quantity of food consumed is too low to induce a toxic effect. The amount of pesticides detected, and which is gradually consumed by the bees, seems to induce subclinical effects. There are no available data on decreasing the quantity of pesticides stored in pollen loads or bee bread. Our results indicate, however, that insecticide residues remain in pollen loads when using doses appropriate for crop protection purposes, and regularly contaminate the grass and undergrowth in orchards. Growers apply pesticides at night when honey bees are inside their hives, thus allowing degradation of the chemicals in the environment to minimise possible pollen contamination or honey bee intoxication. Frequent advice to protect honey bees from contact with insecticides could help to ensure healthy honey bee colonies and protect other important pollinators from poisoning. Further research is needed in order to establish all possible sublethal effects on individual honey bees and brood of the quantities of pesticides present in the pollen collected from treated fields.

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