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Does Cry1Ab protein affect learning performances of the honey bee *Apis mellifera* L. (Hymenoptera, Apidae)?☆

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Abstract

Genetically modified Bt crops are increasingly used worldwide but side effects and especially sublethal effects on beneficial insects remain poorly studied. Honey bees are beneficial insects for natural and cultivated ecosystems through pollination. The goal of the present study was to assess potential effects of two concentrations of Cry1Ab protein (3 and 5000 ppb) on young adult honey bees. Following a complementary bioassay, our experiments evaluated effects of the Cry1Ab on three major life traits of young adult honey bees: (a) survival of honey bees during sub-chronic exposure to Cry1Ab, (b) feeding behaviour, and (c) learning performance at the time that honey bees become foragers. The latter effect was tested using the proboscis extension reflex (PER) procedure. The same effects were also tested using a chemical pesticide, imidacloprid, as positive reference. The tested concentrations of Cry1Ab protein did not cause lethal effects on honey bees. However, honey bee feeding behaviour was affected when exposed to the highest concentration of Cry1Ab protein, with honey bees taking longer to imbibe the contaminated syrup. Moreover, honey bees exposed to 5000 ppb of Cry1Ab had disturbed learning performances. Honey bees continued to respond to a conditioned odour even in the absence of a food reward. Our results show that transgenic crops expressing Cry1Ab protein at 5000 ppb may affect food consumption or learning processes and thereby may impact honey bee foraging efficiency. The implications of these results are discussed in terms of risks of transgenic Bt crops for honey bees.

Keywords: Sublethal effect; Conditioned response; Proboscis extension response; Feeding behaviour; Imidacloprid

1. Introduction

Genetically modified (GM) crops are becoming an increasingly important feature of agricultural landscapes, Bt corn being one of the most widely grown GM crops worldwide (James, 2004). The use of Bt corn is considered a successful alternative to direct pesticide application against a variety of corn pest insects. Due to its broad use by farmers, it is important to evaluate potential effects of Bt-protein on nontarget species in agro-ecosystems (Andow and Zwahlen, 2006). GM Bt corn MON810 and event176 express the Cry1Ab protein of the soil bacterium *Bacillus thuringiensis* in corn tissues (Fearing et al., 1997). The Cry1Ab protein is targeted against Lepidoptera (Höfte and Whiteley, 1989), particularly *Ostrinia nubilalis* Hübner in corn. In susceptible insects, Cry1Ab crystal proteins produce lesions in the midgut epithelium (Knowles and Dow, 1993) inducing septicemia caused by enteric bacteria of the exposed insects (Broderick et al., 2006).

Bt corn is considered harmless to nontarget organisms outside the order of Lepidoptera and in general, studies

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The experiments were conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

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have reported minimal side effects on beneficial insects (see O'Callaghan et al., 2005). However, risks of exposure and subsequent effects may depend on the nontarget insects considered (Harwood et al., 2005; Andow et al., 2006), and only few studies have investigated sublethal effects of Bt proteins on nontarget insects (Kaiser et al., 2001; Ludy and Lang, 2006; Lang and Vojtech, 2006; Prasifka et al., 2007). Specifically for honey bees, most studies only assess lethal toxicity (see Malone and Pham-Delegue, 2001). Thus, additional insights on the potential effects of Cry1Ab protein on physiological and behavioural components of nontarget insects need to be gained.

Bt corn may constitute a risk for pollinators, such as honey bees, because of the presence of Cry1Ab endotoxin in corn pollen (Fearing et al., 1997; Wraight et al., 2000), and other food sources (Malone and Pham-Delegue, 2001). In *Apis mellifera* L., pollen is a major food for larvae and young bees after emergence (Haydak, 1970). While larval or young adult honey bees are fed pollen, the development of physiological structures involved in olfaction and learning performances takes place (Masson and Arnold, 1984; Masson et al., 1993), and the effects of Cry1Ab proteins on these processes deserve further attention. For example, Babendreier et al. (2005) reported that Bt toxins are found in hypopharyngeal glands of young honey bees that were fed Bt contaminated food. No effect was detected on gland development, but the study showed that Bt toxins could accumulate in honey bee tissues. Exposure of young honey bees to Bt toxins may cause deleterious effects on development and learning performance. Learning is of primary importance in honey bee foraging (Winston, 1987), therefore any indirect and/or direct impairments of this capability can affect the development of honey bee colonies and their role as pollinators in agricultural crops.

The proboscis extension reflex (PER) procedure is a reliable method to quantify effects of chemical pesticides on the learning performances of honey bees (Abramson et al., 1999; Decourtye and Pham-Delègue, 2002; Decourtye et al., 2004a, b; Desneux et al., 2007). This assay has been used to demonstrate negative effects of GM proteinase inhibitors on honey bee learning (Picard-Nizou et al., 1997; Pham-Delègue et al., 2000). The PER assay simulates honey bee–plant interactions and is based on the temporal paired association of a conditioned stimulus and an unconditioned stimulus. During conditioning, the PER is elicited by contacting the gustatory receptors of the antennae with a sucrose solution (unconditioned stimulus), and simultaneously delivering an odour (conditioned stimulus). Extension of the proboscis is immediately rewarded by the uptake of a sucrose solution. Bees can exhibit the PER as a conditioned response to the odour alone after even a single pairing of the odour with a sucrose reward.

The goal of this study was to provide a lethal and sublethal toxicity assessment of the Cry1Ab protein on the honey bee, *A. mellifera*. Our experiments evaluated these effects on three life traits of young honey bee adults: (a) survival of workers bees during sub-chronic exposure to Cry1Ab, (b) feeding behaviour, and (c) learning performances. Tests were conducted on young honey bee adults regarding the known high consumption rate of pollen during the first day of imago life in honey bees (Haydak, 1970) and thus potential Cry1Ab exposure at that time. Oral sub-chronic exposure was chosen to mimic exposure conditions of honey bees feeding on a contaminated food source in the hive. Subsequent impact on feeding behaviour was assessed to detect potential anti-feeding effects of pesticides and Cry1Ab on honey bees. Finally, we assessed the impact of Cry1Ab on learning processes (using PER procedure) because of the importance of these behavioural traits for colony performance (Menzel et al., 1993). We used the pesticide imidacloprid as positive control because previous work reported several deleterious effects on food consumption and learning processes in honey bees (Decourtye et al., 2004a; Ramirez-Romero et al., 2005).

2. Material and methods

2.1. Preparation of contaminated syrups

Uncontaminated, Cry1Ab-contaminated and imidacloprid-contaminated syrups were used in the experiments. The Cry1Ab protein was provided by the INRA laboratory "Unité Génétique Microbienne et Environnement" Guyancourt, France. The Cry1Ab protein (131 kDa) was obtained from the *aizawai* 7.29 strain in the pHTA1 plasmid (Sanchis et al., 1988). Imidacloprid (98% pure) was provided by Cluzeau InfoLabo (Sainte-Foy La Grande, France). Control syrup (uncontaminated syrup) contained sucrose ($500\,g\,l^{-1}$) and 1% solvent (acetone and Na_2CO_3), and formed the basis for the treated syrups.

For the Cry1Ab tests, the sugar syrup ($500\,g\,l^{-1}$) contained the Cry1Ab protein at concentrations (nominal) of $3\,\mu g\,kg^{-1}$ (3 ppb [part per billion]) and $5000\,\mu g\,kg^{-1}$ (5000 ppb). These amounts of Cry1Ab protein are similar to those recorded for pollen of Bt-corn cultivar MON810 ($2\,\mu g\,kg^{-1}$) (Wraight et al., 2000) and pollen of cultivar event176 ($5000\,\mu g\,kg^{-1}$) (Fearing et al., 1997). For the imidacloprid tests, the sugar syrup contained the pesticide at a concentration (nominal) of $48\,\mu g\,kg^{-1}$ (48 ppb). This treatment served as a positive control, as the lowest observed effect doses (LOED) reported on honey bees for mortality and PER were $3.2\,ng\,bee^{-1}\,day^{-1}$ and $0.4\,ng\,bee^{-1}\,day^{-1}$, respectively, (Decourtye et al., 2003) and 48 ppb corresponded to a dose of $0.48\,ng\,bee^{-1}\,day^{-1}$. Fresh syrups were prepared before each experiment and stored at $3\pm1\,^{\circ}C$ in glass vials protected from light.

2.2. Oral sub-chronic exposure

Emerging honey bees were collected from a bee hive's brood comb during summer and were kept in "Pain" cages in groups of 50 individuals (Pain, 1966). The cages were maintained in the dark in an incubator at $33\pm1\,^{\circ}C$, $55\pm5\%$ relative humidity. Following the method described by Decourtye et al. (2004a), bees were provided with pollen the first eight days of their life, and were allowed to feed ad libitum on water and candi sugar (75% sucrose, 25% honey; Haydak, 1970) during the first two days (before exposure to treatments).

When 2 days old, bees were starved for 2 h and then received contaminated or uncontaminated syrup every day during 12 days according to EPPO method 170 (EPPO, 1993). Syrups were offered at a rate of $10\,\mu l\,bee^{-1}\,day^{-1}$. After bees completely consumed the contaminated syrups or uncontaminated syrup (control), the uncontaminated syrup was offered ad libitum until the next day. Contaminated and uncontaminated syrups were offered in 3 ml Eppendorf® tubes with

hermetic lids, and a fine perforation in the bottom (1.5 mm) to let syrup pass. Depending of treatments, two to four replicates were undertaken, with 50 bees per replicate per treatment.

2.3. Mortality and syrup consumption

The rate of consumption of uncontaminated syrup and honey bee mortality was recorded daily at noon. Then after the 2 h starvation period, contaminated syrups were offered until consumption was complete (as described in the previous section), and the time spent to complete consumption was recorded. After total consumption has been observed in all groups, honey bees were fed with uncontaminated syrup. Honey bees were considered dead when they remained completely immobile. All dead bees were removed from the cages. We used feeding duration on the contaminated syrups as indication of treatment effect because Cry1Ab protein is known to reduce food uptake in lepidopteran insects (Schnepf et al., 1998) and chemical pesticides could induce antifeedant effects in treated insects (Desneux et al., 2007).

2.4. Conditioned PER

The experimental procedure for conditioning the proboscis extension was the standard one used in previous studies on olfactory learning in honey bees (Pham-Delègue et al., 1993) and successfully used to assess sublethal effects of pesticides on honey bee learning (Decourtye et al., 2004a, 2005). The tested bees were chosen from the bees surviving the oral sub-chronic exposure. They were tested when they were 14–15 days old, which is the age they would become foragers under natural conditions (Seeley, 1983). Bees of this age exhibit the best learning performances in an olfactory Pavlovien conditioning procedure (Pham-Delègue et al., 1990).

Immediately after the last oral sub-chronic exposure, the bees were mounted individually in glass tubes with only their antennae and mouthparts free. They were starved for 3 h prior to conditioning. They were selected for showing a PER after stimulation of the antennae with a sucrose solution (30%). The number of individuals exhibiting the reflex response was recorded. The ability to produce the reflex response reflects the state of the sensory-motor pathway underlying the PER. Bees were then placed in the main airflow (50 ml s^{-1}) to be familiarized with the experimental context. For the conditioning trials, the conditioned stimulus (10 μl of pure linalool, a common floral odour (Blight et al., 1997), deposited on a filter paper strip inserted in a Pasteur pipette cartridge; Sigma, 95% purity) was delivered through a secondary airflow (2.5 ml s^{-1} for 6 s). During odour delivery, the PER was elicited after 3 s by contacting the antennae with a sucrose solution (30%) as the unconditioned stimulus, and the same solution was immediately given as a reward, before the odour delivery ended. Three conditioning sessions were carried out at 20 min intervals (conditioning phase; C1, C2 and C3). The individuals were then subjected to five test trials (designated "extinction" phase, and noted T1–T5), during which the conditioned stimulus (pure linalool) was delivered for 6 s at 20 min intervals, without the unconditioned stimulus and the reward. The conditioned PER was recorded as a yes-or-no response during the test trials.

2.5. Statistical analysis

Percent mortality at the end of oral sub-chronic exposure to two levels of Cry1Ab protein and imidacloprid were compared to control using chi-square tests with Bonferroni correction for multiple comparisons. Daily mean quantity of uncontaminated syrups and the daily mean time for total consumption of contaminated syrups (10 μl bee^{-1} day^{-1}) in groups exposed to Cry1Ab and imidacloprid were compared to the control group using a Wilcoxon signed rank test. For each treatment, the number of initial reflex responses, the number of responses during PER (conditioning and extinction phases, at each trial) were compared to the number recorded in the control group using chi-square tests with Bonferroni correction for multiple comparisons. All statistical analyses were performed using SPSS (Systat®, Chicago, USA).

3. Results

3.1. Mortality and syrup consumption

Exposure of young adult honey bees to Cry1Ab-contaminated syrups or imidacloprid-contaminated syrup for 12 days did not increase mortality compared to a control group (Table 1; $P>0.05$).

The mean time for total consumption of Cry1Ab-contaminated syrup at the 3 ppb concentration was not significantly different from the time recorded in the control group ($Z=-0.961$; $P=0.337$) (Fig. 1). However, honey bees exposed to Cry1Ab at 5000 ppb spent significantly more time to completely consume the treated syrup ($Z=-2.256$; $P=0.024$). Similar effects were found for honey bees fed on imidacloprid-contaminated syrup ($Z=-2.978$; $P=0.003$).

The consumption of uncontaminated syrup per day was significantly lower in honey bees subjected to sub-chronic exposure of 5000 ppb Cry1Ab compared to those from the control group ($Z=-2.776$; $P=0.006$) (Fig. 2). In contrast, Cry1Ab at 3 ppb did not significantly affect the consumption rate of uncontaminated syrup ($Z=-1.686$; $P=0.092$), nor did imidacloprid when compared to the control group ($Z=-1.120$; $P=0.263$).

3.2. Proboscis extension reflex

The levels of proboscis reflex response in imidacloprid-treated (85%) and Cry1Ab-treated bees (3 ppb: 97.5%, 5000 ppb: 95%) were not significantly different from the control group (93.7%), (all $P>0.05$). This suggests that the exposure to imidacloprid and Cry1Ab protein at the tested doses did not disrupt the sensory and motor components controlling the PER.

After exposure to Cry1Ab protein-contaminated syrups, the honey bees were not significantly affected in the different trials of the conditioning phase (C1–C3) and in the first four trials of the extinction phase (T1–T4) (all $P>0.05$; Fig. 3). However, a significant difference was observed between 5000 ppb Cry1Ab and the control group during the fifth trial of the extinction phase (T5).

Table 1
Percentage mortality in honey bees after a 12-day period of sub-chronic exposure to Cry1Ab at two concentrations (3 ppb [part per billion]; 5000 ppb) and imidacloprid (48 ppb)

	Percentage of mortality	Statistic results
Control	8.0	
Cry1Ab 3 ppb	19.0 NS	$\chi^2=4.507$; $P=0.116$
Cry1Ab 5000 ppb	11.0 NS	$\chi^2=0.278$; $P=0.934$
Imidacloprid	13.0 NS	$\chi^2=0.851$; $P=0.733$

NS: not significantly different from the control (chi-square test; $P>0.05$).

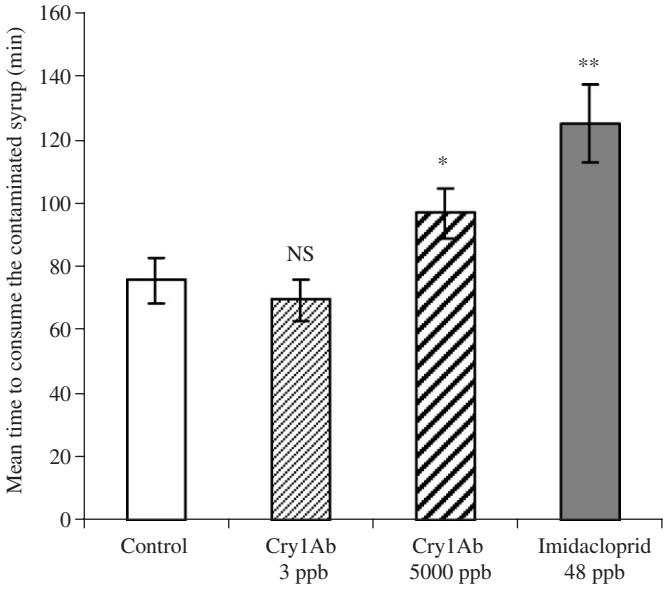

Fig. 1. Mean time (±SE) to completely consume syrup (10 μl bee^{-1}) for honey bees exposed to two Cry1Ab protein concentrations (3 ppb [part per billion]; 5000 ppb), imidacloprid (48 ppb) and the control group. *$P<0.05$; **$P<0.01$; NS: not significant from the control group (Wilcoxon signed rank test).

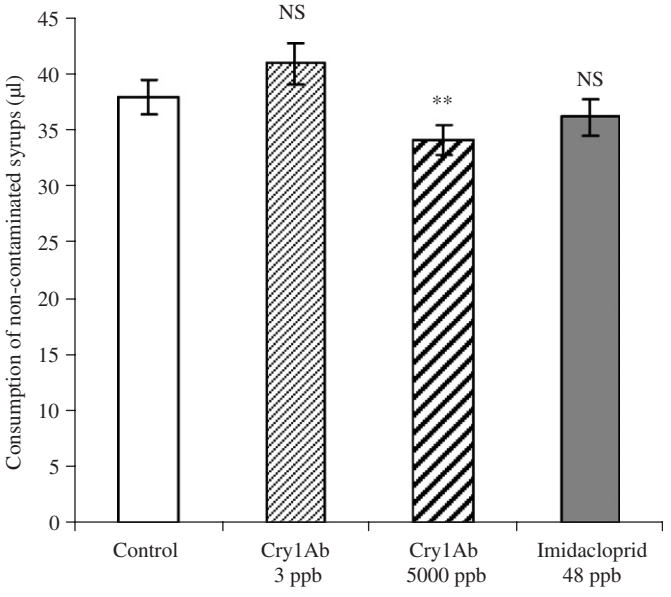

Fig. 2. Mean daily quantity of noncontaminated syrup consumed (±SE) by honey bees from groups subjected to sub-chronic exposure to Cry1Ab protein concentrations (3 ppb; 5000 ppb), imidacloprid (48 ppb) and the control group. **$P<0.01$; NS: not significant from the control group (Wilcoxon signed rank test).

Bees exposed to Cry1Ab syrup showed a significantly higher percentage of response than those from the control group ($\chi^2_1 = 6.721$; $P = 0.028$). This effect was not found for bees exposed to 3 ppb Cry1Ab. In case of exposure to imidacloprid, honey bee responses during the second trial of the conditioning phase (C2) ($\chi^2_1 = 12.457$; $P = 0.001$) were significantly lower than in the control group. Imidacloprid also significantly reduced the percentage of responses of bees at third and fourth trials of the extinction phase (T3: $\chi^2_1 = 11.811$; $P = 0.002$; T4: $\chi^2_1 = 7.201$; $P = 0.022$).

4. Discussion

In pollinators, studies commonly address potential lethal effects of GM products, while sublethal physiological and behavioural effects are poorly studied (see Malone and Pham-Delegue, 2001). Although no lethal effect was observed after oral sub-chronic exposure to Cry1Ab proteins, our study demonstrates behavioural modifications in exposed honey bees. First, honey bees spent more time to completely feed the contaminated syrup at 5000 ppb Cry1Ab, indicating an effect on feeding behaviour. Second, the extinction process during PER was modified after exposure to 5000 ppb Cry1Ab. In that group, 80% of honey bees continued to respond to a conditioned odour in the absence of food reward, while only 50% of bees in the control group responded. For imidacloprid (positive control), the concentration tested was sublethal as expected, and had negative effects on feeding behaviour and on both phases of the PER bioassay. Faster extinction of the conditioned response had been found previously for bees exposed to conventional insecticides (Abramson et al., 1999; Weick and Thorn, 2002; Decourtye et al., 2004b, 2005) and transgenic products (Picard-Nizou et al., 1997; Pham-Delègue et al., 2000). For the first time we demonstrate that pesticide products could also induce higher levels of response during extinction phase.

4.1. Sublethal behavioural effects

Disturbances in the feeding behaviours of bees that received Cry1Ab-contaminated (5000 ppb) or imidacloprid-contaminated syrups may indicate antifeedant effects (Isman, 2006). Such effects have been previously reported for imidacloprid at sublethal doses on Homoptera (Nauen et al., 1998a, b) and honey bees (Decourtye et al., 2003). Moreover, foraging honey bees reduce their visits to foods contaminated by imidacloprid at concentrations above 20 ppb (Kirchner, 1999). In case of Cry1Ab protein, the observed effect was likely an antifeedant effect, as increased feeding time cannot be explained by currently known mechanisms of Cry1Ab action in insects.

Although consumption of uncontaminated syrup was reduced after Cry1Ab treatment, feeding behaviour did not change following exposure to imidacloprid-contaminated syrup. The effect of Cry1Ab suggests that eventual effects are noticeable even after exposure period (in contrast with imidacloprid), but potential mechanisms that underlie such effects need to be explored. An effect of Cry1Ab at the gut level in honey bees would induce lesions and subsequently halt food uptake or cause death as reported in insects sensitive to Cry1Ab (Schnepf et al., 1998). Therefore, our

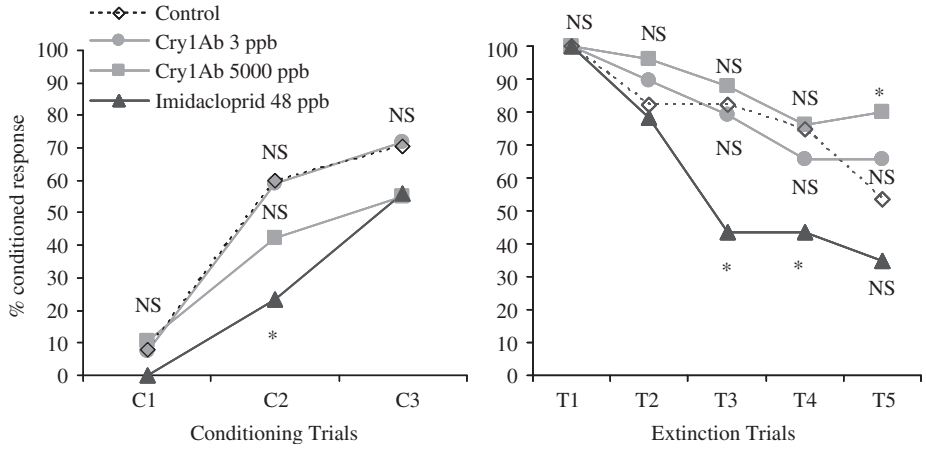

Fig. 3. PER responses of honey bees during conditioning phase (C1–C3) and extinction phase (T1–T5) after sub-chronic exposure to Cry1Ab protein at 3 ppb, (grey lines, rounded plot), 5000 ppb (grey lines, squared plot) and imidacloprid at 48 ppb (black line). Results for control group are also reported (doted lines). $n = 34–41$; $*P<0.05$ indicated a significant difference with the control (chi-square test); NS: not significant (when no treatments differed from the control for a given trial, NS is reported only once).

results are probably not caused by a direct effect at the gut level in honey bees. This hypothesis is supported by recent findings that Cry1Ab does not affect intestinal bacteria in honey bees (Babendreier et al., 2007).

During PER assays, imidacloprid induced perturbations of honey bee responses in both conditioning and extinction phases, as previously reported by Decourtye et al. (2003) and identified at the nervous cellular level (Decourtye et al., 2004a). In our study, Cry1Ab at 3 ppb did not induce any behavioural effects. The 5000 ppb concentration did not disturb conditioning phase showing that honey bees were able to learn the conditioned stimulus correctly (also shown with 1000 ppb Cry1Ab Ramirez-Romero et al., 2005), but induced prolonged retention of responses during the extinction phase. During memorization of a stimulus, different steps are involved: (1) information is stored in the short-term memory during the conditioning phase and (2) extinction phase corresponds to long-term memory (from the model of memory temporal schedule in the honey bee Greggers and Menzel, 1993). Imidacloprid affects the first step of information storage and interferes with the consolidation of the memory (long-term memory); probably due to physiological effects found on cerebral neuropiles (Decourtye et al., 2004a) which have an essential role in olfactory memory (Fahrbach, 2006). But previous studies showed that conditioning and extinction are two independent processes that could be differentially affected by exposure to toxins (Decourtye and Pham-Delègue, 2002; Pham-Delègue et al., 2002). We find here a new case illustrating this fact because Cry1Ab modifies the extinction process but not the conditioning process.

As reflex responses were not affected, observed effects are unlikely to result from disturbances of the sensory–motor process of PER rather than at the central nervous system (memorization process). Our research demonstrates the impact of Cry1Ab on conditioned PER, a complex response which depends on sucrose sensitivity, odour learning and memorization processes (Menzel et al., 1993). Further work is still needed to quantify the effects of Cry1Ab on these different parameters, as investigated previously for imidacloprid using cytochrome oxidase histochemistry procedures (Decourtye et al., 2004a) or other techniques like optical calcium imaging procedures (Joerges et al., 1997). Olfactory-conditioned PER is a standard technique to assess the sublethal effect of neurotoxic pesticides on the olfactory learning abilities of the honey bee (Lambin et al., 2001; Decourtye et al., 2004b; El Hassani et al., 2005). However, it does not allow us to distinguish effects resulting from the direct neurotoxic activity of the compounds from potential indirect effects of the compounds on the behaviour of insects. Although our results do not allow us to identify the precise causes of the effects, they show the potential effect of sublethal doses of Cry1Ab on learning performance in honey bees.

4.2. Implications for risk assessment

The Cry1Ab protein did not induce lethal effect on honey bees meaning no drastic impact at colony scale (in concordance with Ramirez-Romero et al., 2005). However, our work indicated the likelihood of sublethal effects when honey bees are exposed to Cry1Ab foods. Specifically, the Cry1Ab may have an antifeedant effect when present at high concentrations (e.g. 5000 ppb), and may affect learning. In terms of foraging optimality, the extinction process is crucial for exploitation of food resources because it enables foraging honey bees to leave depleted food sources (Herrera, 1990). As Cry1Ab may impair behavioural plasticity of honey bees, their foraging may not be optimal. As a result, foragers could spend more time foraging in sub-optimal or depleted food sources instead of exploring new ones. However, results on PER extinction remain difficult to extrapolate to field conditions. Thus, further studies are still needed to investigate the

mechanisms underlying the effects and the ecological significance of observed effect on PER extinction.

The significance of the observed behavioural perturbations may depend on the real risk of exposure of honey bees to corn pollen containing the Bt protein. The use of contaminated syrups instead of Bt corn pollen in this study might have led to overestimation of the realistic exposure of bees to Cry1Ab, as the bioavailability of this protein is possibly higher in syrups than in pollen. However, because honey bees usually digest maize pollen at a high rate (up to 75%) (Babendreier et al., 2004), exposure of bees to Cry1Ab protein via pollen consumption is probably of the same order as via the consumption of syrups used in our experiments. In addition, Cry1Ab concentrations that invoke sublethal effects may be reached in food stocks of the hive, as continuous food storage may tend to concentrate toxic products within the hive (Villa et al., 2000). To determine honey bee exposure and contamination to Bt proteins under natural conditions, we can use the model detailed by Rortais et al. (2005). In natural conditions, nurse-aged bees consume large amounts of pollen until they become foragers (Hrassnigg and Crailsheim, 1998). Bees ingest an average of 6.5 mg of pollen per day (Crailsheim et al., 1992; Rortais et al., 2005), therefore about 78 mg in 12 days (duration of exposure in our bioassay). The pollen in the hive is composed of a mixture of pollen from different plant species present in the environment, but in areas of extensive monocultures, during the flowering time of the corn, 80% of the total amount of pollen collected by honey bees can be from corn (Odoux et al., 2004; Sabugosa-Madeira et al., 2007). Therefore, a nurse may consume 62.4 mg of corn pollen in 12 days (80% of 78 mg). If this pollen comes from modified Bt corn "event176", each nurse will ingest a maximum of 312 ng of Cry1Ab protein in 12 days ($62.4\,\text{mg} \times 5000\,\mu\text{g}\,\text{kg}^{-1}$). When that dose is compared with our observed effect dose ($5000\,\text{ppb} = 600\,\text{ng}\,\text{bee}^{-1}$ in 12 days), it seems that drastic impact on colony performance is unlikely.

Our results highlight the importance of developing studies assessing how exposures to transgenic crops can affect honey bee foraging capacities. It is especially important because preservation and accumulation of toxins coming from GM crops in the hive is not investigated when assessing the risk of GM crop for pollinators. In the case of Bt corn, however, given the difference between the expected maximum dose of exposure under natural conditions (potential maximum accumulation into the hive) and the observed dose effect (5000 ppb), our general conclusion is that negative effects of Cry1Ab protein on foraging behaviour of honey bees are unlikely in natural conditions.

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References

Abramson, C.I., Aquino, I.S., Ramalho, F.S., Price, J.M., 1999. The effect of insecticides on learning in the Africanized honey bee (*Apis mellifera* L.). Arch. Environ. Contam. Toxicol. 37, 529–535.

Andow, D.A., Zwahlen, C., 2006. Assessing environmental risks of transgenic plants. Ecol. Lett. 9, 196–214.

Andow, D.A., Lovei, G.L., Arpaia, S., 2006. Ecological risk assessment for Bt crops. Nat. Biotechnol. 24, 749–751.

Babendreier, D., Kalberer, N., Romeis, J., Fluri, P., Bigler, F., 2004. Pollen consumption in honey bee larvae: a step forward in the risk assessment of transgenic plants. Apidologie 35, 293–300.

Babendreier, D., Kalberer, N.M., Romeis, J., Fluri, P., Mulligan, E., Bigler, F., 2005. Influence of Bt-transgenic pollen, Bt-toxin and protease inhibitor (SBTI) ingestion on development of the hypopharyngeal glands in honeybees. Apidologie 36, 585–594.

Babendreier, D., Joller, D., Romeis, J., Bigler, F., Widmer, F., 2007. Bacterial community structures in honeybee intestines and their response to two insecticidal proteins. FEMS Microbiol. Ecol. 59, 600–610.

Blight, M.M., Le Métayer, M., Pham-Delègue, M.H., Pickett, M.H., Marion-Poll, F., Wadhams, L.J., 1997. Identification of floral volatiles involved in recognition of oilseed rape flowers, *Brassica napus* by honeybees, *Apis mellifera*. J. Chem. Ecol. 23, 1715–1727.

Broderick, N.A., Raffa, K.F., Handelsman, J., 2006. Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. Proc. Natl. Acad. Sci. USA 103, 15196–15199.

Crailsheim, K., Schneider, L., Hrassnigg, N., Bühlmann, G., Brosch, U., Gmeinbauer, R., Schöffmann, B., 1992. Pollen consumption and utilization in worker honeybees (*Apis mellifera* carnica): dependence on individual age and function. J. Insect Physiol. 38, 409–419.

Decourtye, A., Pham-Delègue, M.H., 2002. The proboscis extension response. Assessing the sublethal effects of pesticides on the honey bee. In: Devillers, J., Pham-Delègue, M.H. (Eds.), Honey Bees: Estimating the Environmental Impact of Chemicals. Taylor & Francis, New York, pp. 67–81.

Decourtye, A., Lacassie, E., Pham-Delègue, M.H., 2003. Learning performances of honeybees (*Apis mellifera* L) are differentially affected by imidacloprid according to the season. Pest Manage. Sci. 59, 269–278.

Decourtye, A., Armengaud, C., Renou, M., Devillers, J., Cluzeau, S., Gauthier, M., Pham-Delègue, M.H., 2004a. Imidacloprid impairs memory and brain metabolism in the honeybee (*Apis mellifera* L.). Pestic. Biochem. Phys. 78, 83–92.

Decourtye, A., Devillers, J., Cluzeau, S., Charreton, M., Pham-Delègue, M.H., 2004b. Effects of imidacloprid and deltamethrin on associative learning in honeybee under semi-field and laboratory conditions. Ecotox. Environ. Safe. 57, 410–419.

Decourtye, A., Devillers, J., Genecque, E., Le Menach, K., Budzinski, H., Cluzeau, S., Pham-Delègue, M.H., 2005. Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honeybee *Apis mellifera*. Arch. Environ. Contam. Toxicol. 48, 242–250.

Desneux, N., Decourtye, A., Delpuech, J.M., 2007. The sublethal effects of pesticides on beneficial arthropods. Annu. Rev. Entomol. 52, 81–106.

El Hassani, A.K., Dacher, M., Gauthier, M., Armengaud, C., 2005. Effects of sublethal doses of fipronil on the behavior of the honeybee (*Apis mellifera*). Pharmacol. Biochem. Behav. 82, 30–39.

EPPO, 1993. Guideline on test methods for evaluating the side-effects of plant protection products on honeybees. EPPO Bull. 22, 203–215.

Fahrbach, S.E., 2006. Structure of the mushroom bodies of the insect brain. Annu. Rev. Entomol. 51, 209–232.

Fearing, P.L., Brown, D., Vlachos, D., Meghji, M.R., Privalle, L., 1997. Quantitative analysis of Cry1Ab expression in Bt maize plants, tissues, and silage and stability of expression over successive generations. Mol. Breeding 3, 169–176.

Greggers, U., Menzel, R., 1993. Memory dynamics and foraging strategies of honeybees. Behav. Ecol. Sociobiol. 32, 17–29.

Harwood, J.D., Wallin, W.G., Obrycki, J.J., 2005. Uptake of Bt endotoxins by nontarget herbivores and higher order arthropod predators: molecular evidence from a transgenic corn agroecosystem. Mol. Ecol. 14, 2815–2823.

Haydak, M.H., 1970. Honey bee nutrition. Annu. Rev. Entomol. 15, 143–156.

Herrera, C.M., 1990. Daily patterns of pollinator activity, differential pollinating effectiveness, and floral resource availability, in a summer-flowering Mediterranean shrub. Oikos 58, 277–288.

Höfte, H., Whiteley, H.R., 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53, 242–255.

Hrassnigg, N., Crailsheim, K., 1998. The influence of brood on the pollen consumption of worker bees (*Apis mellifera* L.). J. Insect. Physiol. 44, 393–404.

Isman, M.B., 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annu. Rev. Entomol. 51, 45–66.

James, C., 2004. Global status of commercialised biotech/GM crops: 2004. ISAAA Briefs, No. 32. International Service for the Acquisition of Agri-biotech Applications, Ithaca, New York.

Joerges, J., Kuttner, A., Galizia, C.G., Menzel, R., 1997. Representations of odours and odour mixtures visualized in the honeybee brain. Nature 387, 285–288.

Kaiser, L., Pham-Delègue, M.H., Ramirez-Romero, R., 2001. Bt corn and insect helpers. Biofutur 207, 30–33.

Kirchner, W.H., 1999. Mad-bee-disease? Sublethal effects of Imidacloprid (''Gaucho'') on the behavior of honey-bees. Apidologie 30, 422.

Knowles, B.H., Dow, J.A.T., 1993. The crystal delta-endotoxins of *Bacillus thuringiensis*—models for their mechanism of action on the insect gut. Bioessays 15, 469–476.

Lambin, M., Armengaud, C., Raymond, S., Gauthier, M., 2001. Imidacloprid induced facilitation of the proboscis extension reflex habituation in the honeybee. Arch. Insect Biochem. Physiol. 48, 129–134.

Lang, A., Vojtech, E., 2006. The effects of pollen consumption of transgenic Bt maize on the common swallowtail, *Papilio machaon* L. (Lepidoptera, Papilionidae). Basic Appl. Ecol. 7, 296–306.

Ludy, C., Lang, A., 2006. Bt maize pollen exposure and impact on the garden spider, *Araneus diadematus*. Entomol. Exp. Appl. 118, 145–156.

Malone, L.A., Pham-Delegue, M.H., 2001. Effects of transgene products on honey bees (*Apis mellifera*) and bumblebees (*Bombus* sp.). Apidologie 32, 287–304.

Masson, C., Arnold, G., 1984. Ontogeny, maturation and plasticity of the olfactory system in the worker bee. J. Insect Physiol. 30, 7–14.

Masson, C., Pham-Delègue, M.H., Fonta, C., Gascuel, J., Arnold, G., Nicolas, G., Kerszberg, M., 1993. Recent advances in the concept of adaptation to natural odor signals in the honeybee, *Apis mellifera* L. Apidologie 24, 169–194.

Menzel, R., Greggers, U., Hammer, M., 1993. Functional organization of appetitive learning and memory in a generalist pollinator, the honey bee. In: Papaj, D.R., Lewis, A.C. (Eds.), Insect Learning. Chapman & Hall, New York, pp. 79–125.

Nauen, R., Hungenberg, H., Tollo, B., Tietjen, K., Elbert, A., 1998a. Antifeedant effect, biological efficacy and high affinity binding of imidacloprid to acetylcholine receptors in *Myzus persicae* and *Myzus nicotianae*. Pestic. Sci. 53, 133–140.

Nauen, R., Koob, B., Elbert, A., 1998b. Antifeedant effects of sublethal dosages of imidacloprid on *Bemisia tabaci*. Entomol. Exp. Appl. 88, 287–293.

O'Callaghan, M., Glare, T.R., Burgess, E.P.J., Malone, L.A., 2005. Effects of plants genetically modified for insect resistance on nontarget organisms. Annu. Rev. Entomol. 50, 271–292.

Odoux, J.F., Lamy, L., Aupinel, P., 2004. L'abeille, récolte-t-elle du pollen de maïs et de tournesol? La Santé de l'Abeille 201, 187–193.

Pain, J., 1966. Nouveau modèle de cagettes expérimentales pour le maintien d'abeilles en captivité. Ann. Abeille 9, 71–76.

Pham-Delègue, M.H., De Jong, R., Masson, C., 1990. Age-dependency of the conditioned proboscis extension response in honeybees. Cr. Acad. Sci. III-Vie 310, 527–532.

Pham-Delègue, M.H., Bailez, O., Blight, M.M., Masson, C., Picard-Nizou, A.L., Wadhams, L.J., 1993. Behavioral discrimination of oilseed rape volatiles by the honeybee *Apis mellifera* L. Chem. Senses 18, 483–494.

Pham-Delègue, M.H., Girard, C., Le Metayer, M., Picard-Nizou, A.L., Hennequet, C., Pons, O., Jouanin, L., 2000. Long-term effects of soybean protease inhibitors on digestive enzymes, survival and learning abilities of honeybees. Entomol. Exp. Appl. 95, 21–29.

Pham-Delègue, M.H., Decourtye, A., Kaiser, L., Devillers, J., 2002. Behavioural methods to assess the effects of pesticides on honey bees. Apidologie 33, 425–432.

Picard-Nizou, A.L., Grison, R., Olsen, L., Pioche, C., Arnold, G., Pham-Delègue, M.H., 1997. Impact of proteins used in plant genetic engineering: toxicity and behavioral study in the honeybee. J. Econ. Entomol. 90, 1710–1716.

Prasifka, P.L., Hellmich, R.L., Prasifka, J.R., Lewis, L.C., 2007. Effects of Cry1Ab-expressing corn anthers on the movement of monarch butterfly larvae. Environ. Entomol. 36, 228–233.

Ramirez-Romero, R., Chaufaux, J., Pham-Delègue, M.H., 2005. Effects of Cry1Ab protoxin, deltamethrin and imidacloprid on the foraging activity and the learning performances of the honeybee *Apis mellifera*, a comparative approach. Apidologie 36, 601–611.

Rortais, A., Arnold, G., Halm, M.P., Touffet-Briens, F., 2005. Modes of honeybees exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by different categories of bees. Apidologie 36, 71–83.

Sabugosa-Madeira, B., Abreu, I., Ribeiro, H., Cunha, M., 2007. Bt transgenic maize pollen and the silent poisoning of the hive. J. Apic. Res. 46, 57–58.

Sanchis, V., Lereclus, D., Menou, G., Chaufaux, J., Lecadet, M.M., 1988. Multiplicity of delta-endotoxin genes with different insecticidal specificities in *Bacillus thuringiensis aizawai* 7.29. Mol. Microbiol. 2, 393–404.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H., 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62, 775–806.

Seeley, T.D., 1983. Division of labour between scouts and recruits in honeybee foraging. Behav. Ecol. Sociobiol. 11, 287–293.

Villa, S., Vighi, M., Finizio, A., Serrín, G.B., 2000. Risk assessment for honeybees from pesticide-exposed pollen. Ecotoxicology 9, 287–297.

Weick, J., Thorn, R.S., 2002. Effects of acute sublethal exposure to coumaphos or diazinon on acquisition and discrimination of odor stimuli in the honey bee (Hymenoptera: Apidae). J. Econ. Entomol. 9, 227–236.

Winston, M.L., 1987. The Biology of the Honeybee. Harvard University Press, Cambridge, MA.

Wraight, C.L., Zangerl, A.R., Carroll, M.J., Berenbaum, M.R., 2000. Absence of toxicity of *Bacillus thuringiensis* pollen to black swallowtails under field conditions. Proc. Natl. Acad. Sci. USA 97, 7700–7703.