# The effects of Bt Cry1Ah toxin on worker honeybees (Apis mellifera ligustica and Apis cerana cerana)

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**Original article**

# The effects of Bt Cry1Ah toxin on worker honeybees (*Apis mellifera ligustica* and *Apis cerana cerana*)

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**Abstract –** We conducted feeding trials in a laboratory setting to test for possible adverse effects of Cry1Ah toxin mixed thoroughly into sugar syrup (60% *w/v* sucrose solution) at three concentrations (10 μg/mL, 10 ng/mL, and 1 ng/mL) on the survival, pollen consumption, and hypopharyngeal gland mass of *Apis mellifera ligustica* and *Apis cerana cerana*. No significant differences in the survival of *A. mellifera* or *A. cerana* were found among groups fed on sugar syrup with or without Cry1Ah toxin. No significant differences were found in the longevity of *A. mellifera* fed sugar syrup with Cry1Ah toxin compared with the control. No differences were detected in the pollen consumption of *A. mellifera ligustica* and *A. cerana cerana*. No significant differences were found in the hypopharyngeal gland weight of 12-day-old honeybees *A. mellifera ligustica* and *A. cerana cerana* fed on sugar syrup with Cry1Ah toxin compared with the control. The implications of these results are discussed in terms of the risks of transgenic corn crops for honeybees.

***Apis mellifera ligustica* / *Apis cerana cerana* / *Bacillus thuringiensis* / Cry1Ah toxin / risk assessment**

## 1. INTRODUCTION

The cultivation of insect-resistant transgenic maize expressing insecticidal crystal proteins from *Bacillus thuringiensis* (Bt) has increased in recent years (James 2009). Non-target effects must be evaluated as part of the environmental risk assessment necessary for the commercialization of transgenic crops (Romeis et al. 2008; Lovei et al. 2009; Desneux and Bernal 2010; Then 2010; Romeis et al. 2011). Honeybees are economically significant pollinators of a number of crops, and they are widely considered as beneficial insects in many natural and agro-ecosystems. Thus, the honeybee may serve as one of the key species to be tested for the potential effects of transgenic crops (Huang et al. 2004).

Bees could be exposed to insecticidal proteins from transgenic plants during foraging. Pollen feeding is likely to be the main route of exposure (Babendreier et al. 2005). Many studies have been conducted to evaluate the side effects of transgenic products on honeybees (O'Callaghan et al. 2005; Malone and Pham-Delègue 2001; Duan et al. 2008). Only a few of those studies, particularly for Bt toxins that target lepidopteran

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pests, show negative effects on honeybees. A review of the sublethal effects reported in the published literature has characterized the different types of sublethal effects of insecticidal compounds on honeybees and described the methods used in these studies (Desneux et al. 2007). Sublethal effects have been increasingly considered in the risk assessment of transgenic crops because of their potential negative effects on bees. The parameters observed included the following: larval survival and pupal dry weight (CryIIIB: Arpaia 1996); mortality (Cry1Ac: Sims 1995; Liu et al. 2009); longevity and food consumption rate (Cry1Ba: Malone et al. 1999; Cry1Ac: Han et al. 2010a); the timing of first flights and the duration of flight activity (Cry1Ba: Malone et al. 2001); foraging activity and learning performance (Cry1Ab: Ramirez-Romero et al. 2005; Ramirez-Romero et al. 2008; Han et al. 2010b); the development of the hypopharyngeal glands (Cry1Ab: Babendreier et al. 2005; Malone et al. 2004); and the intestinal bacterial community (Cry1Ab: Babendreier et al. 2007; Cry1Ac: Jiang et al. 2010). Some semi-field studies involving the use of pollen instead of purified transgene proteins have been conducted using honeybee colonies under open or caged apicultural conditions (Babendreier et al. 2004; Hanley et al. 2003). Relatively few large-scale field studies have been conducted to assess the possible ecological impact of transgenic crops on honeybee colonies under realistic apicultural conditions (Huang et al. 2004; Babendreier et al. 2004; Rose et al. 2007).

Toxicity tests with purified transgene products are often the first step in assessing the risks to honeybees from transgenic crop plants (O'Callaghan et al. 2005). Pre-release honeybee biosafety tests have been conducted for each Bt crop registered. Each test involved feeding bee adults with purified Cry proteins in sucrose solutions at concentrations that greatly exceeded those recorded from the pollen or nectar of the genetically modified (GM) plants in question (O'callaghan et al. 2005). *Apis mellifera ligustica* and *Apis cerana cerana* are the most important bee species in China. Since the introduction of *A. mellifera* in the twentieth century, the quantity and distribution of *A. cerana* in China have decreased significantly from their historic values (Yang 2005). In some localities, this important Chinese bee species is endangered and risks extinction. The use of transgenic plants may pose additional risks for this rare species. The *cry1Ah* gene was cloned, and its expression product has a higher toxicity to *Ostrinia furnacalis* than those of the *cry1Ac*, *cry1Ab*, and *cry1Ie* genes (Xue et al. 2008a, b). Transgenic maize with the *cry1Ah* gene had a strong effect against *O. furnacalis* larvae both in the laboratory and in the field and had potential commercialization prospects (Wang et al. 2008).

The objective of this study was to assess the lethal and sublethal effects of various concentrations of the Cry1Ah toxin on two honeybee species, *A. mellifera ligustica* and *A. cerana cerana*. We evaluated the potential side effects of the toxin on the survival, pollen consumption, and development of the hypopharyngeal gland in the bees. The hypopharyngeal glands are an important aspect of the bee life history and play a significant role in the development of the whole colony.

## 2. MATERIALS AND METHODS

Stocks of *A. mellifera* and *A. cerana* were obtained from our apiary at the Institute of Apicultural Research, Beijing. Brood frames were placed in an incubator (34±1°C, 60±10% relative humidity, darkness) after the cells were capped at approximately 9 days. Newly emerged bees were assigned randomly to wooden cages (9×9×10 cm) with mesh on two sides. A total of 30 bees were used per cage. All bees used in the experiments were less than 12 h old. Each cage was fitted with a gravity feeder containing sugar syrup (60% *w/v* sucrose solution). Sufficient pollen-food was prepared (0.67 parts pollen and 0.33 parts sucrose mixed with water) to supply the total number of bees in each treatment group. The pollen was bee-collected and stored at −20°C.

Cry1Ah toxin was mixed thoroughly into sugar syrup (60% *w/v* sucrose solution) at three concentrations, 1 ng/mL, 10 ng/mL, and 10 μg/mL. The high-concentration Cry1Ah toxin treatment in our experiments represents the worst case. Activated Cry1Ah toxin obtained from a large-scale fermentation of *B. thuringiensis* Bt8 was supplied by the State Key Laboratory for Biology of Plant Diseases and Insect

Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing. Activated toxin was used because this form of the toxin most closely resembles the form in which Cry1Ah will be expressed in transgenic maize (Wang et al. 2008). For each cage, 2 g of pollen-food was supplied. This food was weighed and replaced with fresh pollen-food every 3 days for 21 days. The numbers of surviving bees in each cage were recorded and dead bees removed every day.

Each cage had fresh pollen-food provided. Gland weight is at a maximum in 12-day-old bees (Fluri et al. 1982; Babendreier et al. 2005). The hypopharyngeal glands of 12-day-old bees were dissected in sodium chloride solution (0.25 M). For each treatment, we dissected 30 bees and measured the hypopharyngeal gland weight of each bee.

Significant differences among the treatments in longevity, pollen consumption, and hypopharyngeal gland weight were tested using a one-way analysis of variance (ANOVA). If significant differences were found ($P<0.05$), multiple comparison procedures were performed with Duncan's multiple-range test. Survival was tested using the Kaplan–Meier estimator. All statistical analyses were performed using SAS™ (Cary, NC, USA) (SAS Institute 2000).

## 3. RESULTS

### 3.1. Survival

The results indicated that *A. mellifera* fed on sugar syrup without Cry1Ah died within 61 days, whereas bees fed on sugar syrup with 1 ng/mL, 10 ng/mL, and 10 μg/mL Cry1Ah died within 59, 56, and 59 days, respectively (Figure 1a). *A. cerana* fed on sugar syrup without Cry1Ah died within 44 days, whereas bees fed sugar syrup with 1 ng/mL, 10 ng/mL, and 10 μg/mL Cry1Ah died within 45, 45, and 47 days, respectively (Figure 1b). The survival curve shows that the survival (percent living) of *A. mellifera* and *A. cerana* in the control group did not differ significantly from that of groups fed sugar syrup with 1 ng/mL, 10 ng/mL, or 10 μg/mL Cry1Ah toxin ($\chi^2=7.3715$, $df=3$, $P=0.0610$ for *A. mellifera*; $\chi^2=1.2324$, $df=3$, $P=0.2669$ for *A. cerana*; Figure 1a, b). No significant differences were found in the longevity of *A. mellifera* fed on sugar syrup with Cry1Ah toxin compared with a control treatment in which bees were fed sugar syrup with no Cry1Ah toxin ($F=2.28$, $df=588$, $P=0.0787$; Figure 2). Significant differences in the longevity of *A. cerana cerana* were found among the groups tested ($F=6.25$, $df=606$, $P=0.0003$; Figure 2). No significant differences were found in the longevity of *A. cerana* fed on sugar syrup with 1 ng/mL or 10 μg/mL Cry1Ah toxin compared with the control treatment, but the longevity of bees fed on sugar syrup with 10 ng/mL Cry1Ah toxin was significantly longer than the control.

### 3.2. Pollen consumption

*A. mellifera* did not consume pollen-food after 21 days, and *A. cerana* did not consume pollen-food after 18 days. No significant differences were found in the pollen consumption of honeybees fed on sugar syrup with 1 ng/mL, 10 ng/mL, or 10 μg/mL Cry1Ah toxin compared with a control treatment in which bees were fed on sugar syrup with no Cry1Ah toxin (all $P>0.05$; Table I).

### 3.3. Hypopharyngeal gland weight

No significant differences were found in the hypopharyngeal gland weight of 12-day-old honeybees *A. mellifera ligustica* and *A. cerana cerana* fed on sugar syrup with 1 ng/mL, 10 ng/mL, or 10 μg/mL Cry1Ah toxin compared with a control treatment in which bees were fed sugar syrup with no Cry1Ah toxin ($F=0.47$, $df=119$, $P=0.7030$ for *A. mellifera*; $F=1.63$, $df=119$, $P=0.1852$ for *A. cerana*; Figure 3).

## 4. DISCUSSION

In this study, we investigated the effects of Cry1Ah toxin on the survival, pollen consumption, and the hypopharyngeal gland weight of *A. mellifera ligustica* and *A. cerana cerana* under laboratory conditions.

The Cry1Ah protein (1 ng/mL, 10 ng/mL, and 10 μg/mL) did not have any lethal effects on *A. mellifera*. The findings of the present study

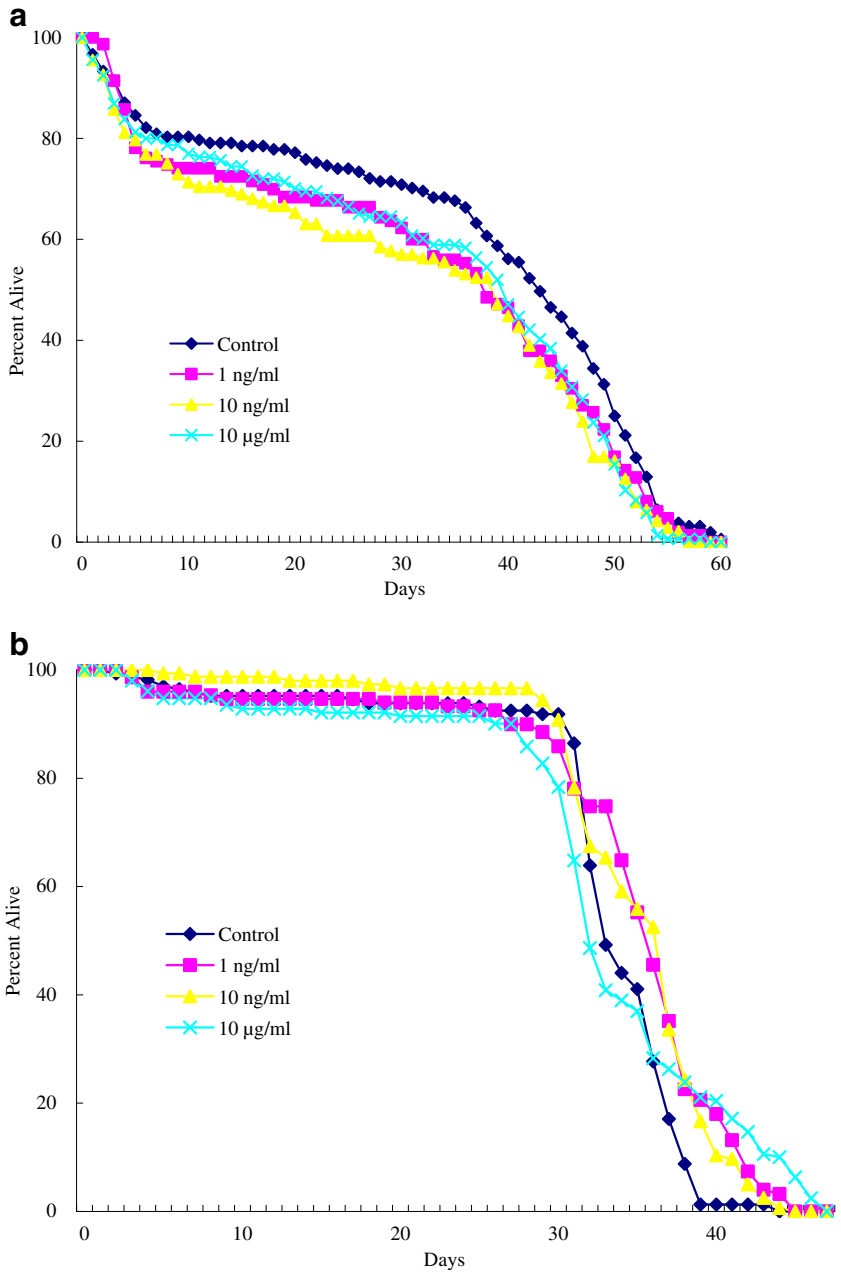


**Figure 1.** Survival of honeybees *A. mellifera ligustica* (**a**) and *A. cerana cerana* (**b**) fed on sugar syrup containing 1 ng/mL, 10 ng/mL, and 10 µg/mL Cry1Ah Bt toxin and without toxin.

regarding mortality and longevity are consistent with those of previous studies (Arpaia 1996; Malone et al. 2004; Ramirez-Romero et al. 2005; Malone and Pham-Delègue 2001; Duan et al. 2008; Malone et al. 1999; Sims 1995). No significant differences were found in the longevity of *A. cerana* fed on sugar syrup with 1 ng/mL or 10 µg/mL Cry1Ah toxin compared with the

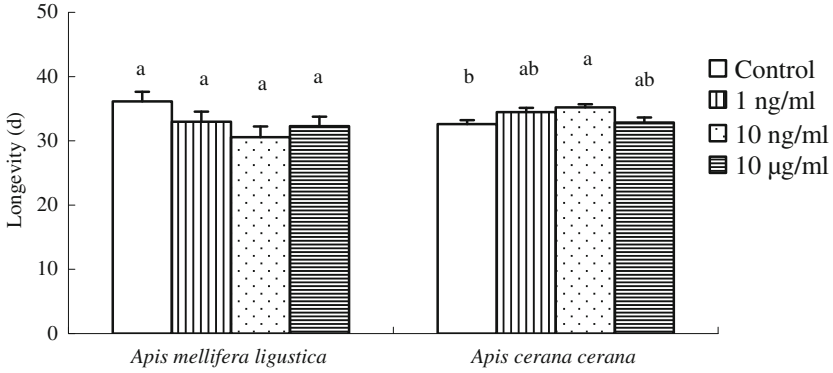


**Figure 2.** Longevity of honeybees *A. mellifera ligustica* and *A. cerana cerana* fed on sugar syrup containing 1 ng/mL, 10 ng/mL, and 10 μg/mL Cry1Ah Bt toxin and without toxin. *Mean bars* with SE bearing the *same letter* are not significantly different at the 5% probability level.

control treatment, but the longevity of bees fed on sugar syrup with 10 ng/mL Cry1Ah toxin was significantly longer than that found for the control.

Pollen consumption was observed after oral exposure to Cry1Ah toxin. Our study found no differences in food consumption compared with the control for exposed honeybees *A. mellifera ligustica* and *A. cerana cerana*. No side effects on the food consumption of honeybees were observed in earlier studies (Cry1Ba: Malone et al. 1999; Cry1Ab: Rose et al. 2007). A change in food consumption has recently been reported. Honeybees took more time to completely consume contaminated syrup at a concentration

**Table I.** Mean quantity of pollen consumed (±SE) by honeybees *A. mellifera ligustica* and *A. cerana cerana* fed with sugar syrup containing 1, 10, and 10,000 ng/ml of Cry1Ah Bt toxin and without toxin.

| | Days | Consumption of pollen per bee (±SE) (mg) | | | | *F* | *df* | *P* |
|---|---|---|---|---|---|---|---|---|
| | | Control | 1 ng/ml | 10 ng/ml | 10,000 ng/ml | | | |
| *Apis mellifera ligustica* | 1–3 | 8.46±2.75 | 9.19±2.39 | 7.39±2.64 | 9.50±2.44 | 0.27 | 19 | 0.8476 |
| | 4–6 | 7.02±1.09 | 9.19±2.92 | 10.71±2.56 | 10.43±2.87 | 1.89 | 19 | 0.1739 |
| | 7–9 | 5.98±0.37 | 7.40±2.44 | 6.43±1.20 | 6.63±2.26 | 0.48 | 19 | 0.7019 |
| | 10–12 | 6.15±0.55 | 7.36±1.26 | 6.82±1.36 | 6.61±1.04 | 0.31 | 19 | 0.8173 |
| | 13–15 | 5.71±1.09 | 6.04±2.04 | 6.09±1.53 | 5.27±0.77 | 0.33 | 19 | 0.8017 |
| | 16–18 | 3.27±1.07 | 3.02±1.28 | 2.29±0.29 | 2.60±1.53 | 0.67 | 19 | 0.5838 |
| | 19–21 | 1.21±0.64 | 0.39±0.98 | 0.00±1.22 | 0.94±1.33 | 1.24 | 19 | 0.3291 |
| | Sum | 37.80±5.61 | 42.59±12.64 | 39.72±4.99 | 41.98±4.76 | 0.35 | 19 | 0.7885 |
| *Apis cerana cerana* | 1–3 | 17.00±1.84 | 15.51±1.80 | 14.79±2.31 | 20.49±0.96 | 1.99 | 19 | 0.1561 |
| | 4–6 | 10.47±1.14 | 11.48±0.48 | 10.60±2.36 | 12.74±1.37 | 0.49 | 19 | 0.6957 |
| | 7–9 | 10.49±0.27 | 11.35±0.44 | 12.00±0.62 | 9.40±0.79 | 2.8 | 19 | 0.0736 |
| | 10–12 | 8.72±0.63 | 7.17±0.51 | 9.29±0.68 | 8.45±0.92 | 1.64 | 19 | 0.2207 |
| | 13–15 | 4.93±0.52 | 4.68±0.45 | 5.58±0.80 | 4.79±0.82 | 0.37 | 19 | 0.7789 |
| | 16–18 | 2.23±0.42 | 1.07±0.42 | 1.92±0.74 | 2.01±0.46 | 0.94 | 19 | 0.4456 |
| | Sum | 53.83±2.98 | 51.26±1.25 | 54.18±3.10 | 57.87±3.29 | 0.96 | 19 | 0.4343 |

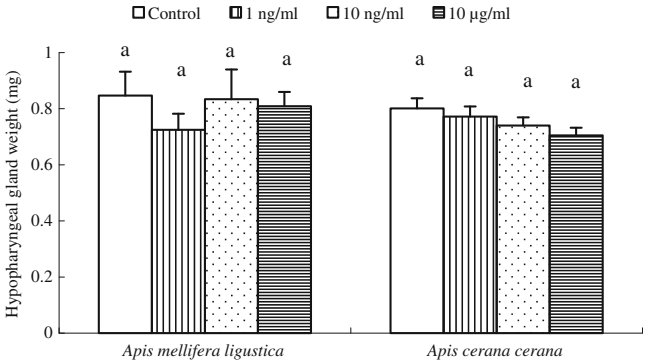


**Figure 3.** Hypopharyngeal gland weight of 12-day-old honeybees *A. mellifera ligustica* and *A. cerana cerana* fed on sugar syrup containing 1 ng/mL, 10 ng/mL, and 10 μg/mL Cry1Ah Bt toxin and without toxin. *Mean bars* with SE bearing the *same letter* are not significantly different at the 5% probability level.

of 5,000 ppb Cry1Ab (Ramirez-Romero et al. 2008). During a 7-day oral exposure to the various treatments, honeybee feeding behavior was disturbed and bees consumed significantly less transgenic cotton pollen (Cry1Ac) than in a control group in which bees were exposed to conventional cotton pollen (Han et al. 2010a).

The hypopharyngeal glands of worker bees might be a good indicator of toxic effects in addition to bee mortality because these glands are very important for honeybee colony development (Babendreier et al. 2005). For this reason, gland mass has been used as an indicator to test the effects of various food sources. Cry1Ah toxin treatment did not affect the hypopharyngeal gland mass of *A. mellifera ligustica* and *A. cerana cerana* fed on sugar syrup with Cry1Ah toxin compared with the control. Negative effects of other Bt toxins on the honeybee hypopharyngeal gland have not been reported (Cry1Ab for Babendreier et al. 2005; Cry1Ba for Malone et al. 2004).

Tests were conducted on young honeybee adults to measure effects on the known high consumption rate of pollen (Haydak 1970) and thus the potential Cry1Ah exposure at that time. Although experiments using purified gene products mixed into bee food can potentially provide useful information on the impacts of transgenic plants prior to release, the current lack of comprehensive data on the expression levels of Cry1Ah proteins in transgenic maize pollen means that it remains difficult to estimate the concentrations of transgene products to which bees may be exposed in the field. We conclude that the Cry1Ah toxin did not affect the survival, pollen consumption, or the hypopharyngeal gland mass of honeybees *A. mellifera ligustica* and *A. cerana cerana.* However, an assessment of the side effects of Cry1Ah corn pollen on honeybees should be conducted under more realistic conditions (i.e., bees fed on GM pollen or field trials).

**Les effets de la toxine Bt (Cry1Ah) sur les ouvrières d'abeilles (*Apis mellifera ligustica* et *Apis cerana cerana*)**

***Bacillus thuringiensis* / évaluation des risques / toxicologie / toxine Cry1Ah**

**Effekte von Bt Cry1Ah-Toxin auf Arbeiterinnen der Honigbiene (*Apis mellifera ligustica* und *Apis cerana cerana*)**

***Apis mellifera ligustica* / *Apis cerana cerana* / *Bacillus thuringiensis* / Cry1Ah-Toxin / Risikoabschätzung**

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