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SHORT COMMUNICATION

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First field assessment of *Bacillus thuringiensis* subsp. *kurstaki* aerial application on the colony performance of *Apis mellifera* L. (Hymenoptera: Apidae)

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Abstract

Honeybee populations around the world are experiencing a decrease in colony numbers probably due to a combination of different causes, such as diseases, poor nutrition and frequent applications of insecticides to control pests. Previous studies about the effect of pesticide *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) on *Apis mellifera* L. report different results. The aim of this study was to analyze the effect of field aerial applications of *Btk* on bee colony performance, specifically on the brood cell percentage evolution, which can be used as an indicator of queen health and brood development breeding rates. To achieve it, the brood cell surface was photographed in every sampling, and data were analyzed using a method based on image treatment software. A total of 480 pictures were examined from two groups of four nucleus hives in two areas, one receiving aerial spraying with *Btk* and the other without treatment. A mixed factorial design was realized to analyse the data showing no differences in colony performance between the two groups of colonies either before the treatment, during and at the end of the assay. Furthermore, the brood surface ratio of *Btk*-treated/untreated increased along the experiment. Therefore, the results of the present study suggest that *Btk* aerial applications did not affect the brood development of honeybees under natural conditions. Nevertheless further field studies are required to ascertain a safe use of *Btk* in forest pest management.

Additional key words: honeybee; *Btk*; field study; Balearic Islands; forest management; impact.

Pollination is a crucial process to preserve the ecosystems (Klein *et al.*, 2007). One of most effective pollinators of wild flowers and cultivated crops is the honeybee (*Apis mellifera* Linnaeus), which represents the 60-95% of the overall pollination in some geographical zones (Morse & Calderone, 2000). Nowadays honeybee populations throughout the world have been subjected to rapid losses (Underwood & vanEngelsdorp, 2007; vanEngelsdorp *et al.*, 2009; Neumann & Carreck, 2010; Pohorecka *et al.*, 2011). Some authors confirm that these losses are triggered by different combination of causes, such as diseases (*Varroa destructor*, *Nosema ceranae* and virus), poor nutrition and frequent applications of insecticides to control pests (Miranda *et al.*, 2003; Cox-Foster & VanEngelsdorp, 2009; Higes *et al.*, 2010). In the present study the impact of the pesticide *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) on honeybees was evaluated. There is a lack of studies developed to evaluate the impact of *Btk* aerial application on *A. mellifera,* although there are some studies developed to evaluate the impact on many groups of animals, such as non-target Lepidoptera (Boulton, 2004), non-target soil organisms (Addison & Barker, 2006) and songbirds (Sopuck *et al.*, 2002). The effect on bees have been developed in some field and laboratory studies, although the majority of the experiments tested the toxicology of *Btk* used in genetically modified crops (O'Callaghan *et al.*, 2005; Duan *et al.*, 2008). These studies showed no adverse effect on *A. mellifera* (Malone & Pham-Delègue, 2001; Hanley *et al.*, 2003; Malone *et al.*, 2004; Porcar *et al.*, 2008), however, some authors confirmed a toxicity of *Btk* under controlled conditions (Ramirez-Romero *et al.*, 2005; Brighenti *et al.*, 2007). It is worth to note that it must be considered that the field results using *Btk* aerial application differ from the laboratory experiments and some authors require more information about effect under field apicultural conditions (Rose *et al.*, 2007;

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Duan *et al.*, 2008; Mommaerts *et al.*, 2010). On the other hand, it is often difficult to extrapolate the impact of aerial application from field results obtained in transgenic crops.

In order to confirm or reject whether *Btk* aerial treatment affect to colony performance of honeybees in field conditions, the present assay analyzes the evolution of the percentage of each frame occupied with brood [as an indicator of queen health and brood development (Dai *et al.*, 2012)] under the effect of *Btk* aerial treatment, which is realized with the purpose of controlling the pine processionary caterpillar *Thaumeopoea pityocampa* (Denis & Schiffermüller) in Ibiza (Balearic Islands, Spain).

To this aim, eight Langstroth nucleus hives were located in two pine forests (four nucleus per field) of Ibiza (572.56 km^2), West Mediterranean island. According to Dai *et al.* (2012) methodology, two fields were selected. One of the forests is located in a zone treated with *Btk* (UTM: 31S 379032 m E 4322751 m N), while the second one is in a treatment-free protected area, and was considered as control (UTM: 31S 367720 m E 4321474 m N). The two zones are separated by about 10 km, an insurmountable distance for a bee flight, and also far enough to be free of the spray wind drift. To make sure those bee populations of all nucleus hives were as homogeneous as possible, sister queens from the same breeding line were reared by using Doolittle method (Flores *et al.*, 1998). The frames of all colonies were made from organic wax and bees did not receive any chemical treatment.

A BACI (Before-After Control-Impact) design (Green, 1979) was conducted in this study. The firsts measurements were taken on August 25, 2009, two months before the treatment applied, which was realized by helicopter on October 20, 2009. The product applied was Foray 48 B, Kenogard S.A., Spain (*B. thuringiensis* subsp. *kurstaki*, 11.8% p/v (11.8 · 10^6 of IU g^{-1}). It was a suspension concentrate (SC); 3.5 L ha^{-1} ultra-low volume application, drop diameter: 100-125 microns). By knowing the initial state, the environmental heterogeneity was controlled. First's five samplings were taken fortnightly, except the last sampling, which were taken one month later on December 16, 2009.

Both faces of every frame were photographed in every sampling. A total of 480 pictures were taken and examined. In each sampling, 80 pictures were taken (10 pictures per hive). Each digital photograph was processed with the Image Analysis Software SIG ArcGIS (ESRI), in order to calculate the percentage of cells occupied with brood (open brood as well as capped brood) in relation to the total surface of the frame, as an effective measurement of the bee's brooding efficiency (Dai *et al.*, 2012).

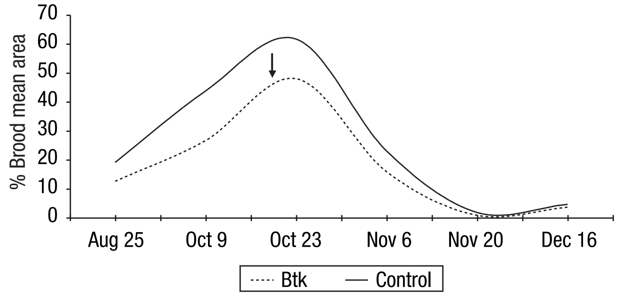

Figure 1. Evolution of the brood area mean percentage of the nucleus hives located in the pine forests, either treated with *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) or untreated, in a study conducted in 2009. Arrow indicates the time of insecticide application.

Data were analysed by a 2 × (6 × 4) mixed factorial design with one between-factor (control/treated groups) and one within-factor (six temporal points) in SPSS v. 20.0 (SPSS Inc., Chicago, IL, USA). A level of $p < 0.05$ was accepted as significant. Means and standard deviations were computed for variable.

As it is shows in Fig. 1, the percentage of brood of both groups of hives showed a strong parallelism throughout the experiment. No significant differences between groups were found (F = 2.59, $p = 0.159$).

During the first three samplings the brood were increased. In the third sampling (just after the *Btk* treatment) the brood area was triplicated in both groups (3.8 fold in *Btk* colonies and 3.2 fold in control hives). In the fourth sampling three colonies of treated site (*Btk1, Btk2* and *Btk4)* and all colonies of control site began to decrease. In the fifth sampling, the brood surface was practically non-existent in six nucleus hives, three of the treated colonies (*Btk1, Btk2 and Btk4*) and three in the control forest zone (control 1, control 2 and control 4). Actually, in both groups there were significant differences between fourth and fifth sampling (t = 4.573, $p = 0.020$ in control group; and t = 3.472, $p = 0.040$ in treated group). In addition to this drastic brood decrement, new queen cells in all of these nucleus hives were observed, as well as the new honeybee swarms in the nearby trees. All of these symptoms suggested that the nucleus hives had lost their queens because of a natural swarming process.

Table 1. Brood mean percentages of the hives analysed during the sampling period in 2009

Hives[1]	Brood mean percentages					
	Before treatment		Post-treatment			
	Aug 25	Oct 9	Oct 23	Nov 6	Nov 20	Dec 16
Btk 1	8.08	8.20	13.31	11.78	0.45	1.18
Btk 2	16.95	53.94	79.93	6.45	0.00	0.00
Btk 3	6.83	12.12	27.82	29.59	2.92	5.12
Btk 4	18.36	32.13	70.89	15.38	0.00	8.30
Btk (M ± SD)	12.56 ± 2.97	26.60 ± 10.51	47.99 ± 16.21	15.80 ± 4.95	0.84 ± 0.70	3.65 ± 1.90
C 1	22.12	55.39	77.60	25.48	0.00	1.20
C 2	21.39	47.11	60.39	15.98	0.00	0.00
C 3	12.23	33.11	55.39	38.98	6.97	8.80
C 4	20.39	39.17	53.21	11.40	0.00	8.35
C M ± SD	19.03 ± 2.30	43.70 ± 4.84	61.65 ± 5.53	22.96 ± 6.09	1.74 ± 1.74	4.59 ± 2.32
Ratio *Btk* / C	0.66	0.61	0.78	0.69	0.48	0.79

[1] *Btk: B. thuringiensis* subsp. *kurstaki;* M: mean; SD: standard deviation; C: control (non-treated nucleus hives).

Although in control 4 there was a low brood percentage, it was observed that all of them remained in a pupal stage without any young or old larvae, which indicates that there was no recent queen laying. Interestingly, in the last sampling, four of these six colonies were recovered (*Btk1, Btk4,* control 1 and control 4) and a similar brood percentage was observed in both hive groups. On the other hand, *Btk* 3 and control 3 were maintained with normal growth (Table 1). At the end of the assay no significant differences between groups of hives were found. None of the nucleus hives showed any disease during the assay, being the reserves of honey and pollen enough to the development of the colonies.

Furthermore, if the brood percentage of both groups are compared through the ratio efficiency *Btk*/control, it can be observed that, even though the *Btk* hives had an initial brood surface smaller than those of the control group, the brood mean ratio increased throughout the experiment: from an initial value of 0.66 (12.56/19.03) to a final 0.79 (3.65/4.59) (Table 1). So the treated group breeding had increased comparatively to the control group breeding.

Unfortunately, we have found very few references on field studies performed under natural conditions, so we can barely compare results. Some authors as Brighenti *et al.* (2007) have reported some mortality rate attributed to *Btk* in laboratory tests involving direct spraying or food poisoning and Ramírez-Romero *et al.* (2005) concluded that *Bt* toxins may have an antifeedant effect in high concentrations. However, it must be considered that insecticide field applications differ from laboratory experiments. On the other hand, this data are in agreement with the majority laboratory studies published based on the effect of *Btk* produced by transgenic farming, which showed that the *Btk* does not affect honeybee populations (Hanley *et al.*, 2003; Malone *et al.*, 2004; Babendreier *et al.*, 2005; O'Callaghan *et al.,* 2005; Duan *et al.*, 2008; Porcar *et al.*, 2008). Furthermore, the results of the present study are consistent with previous works conducted in *Bt* corn pollen (Dai *et al.*, 2012), which showed that the percentage of brood cells did not differ between the *Bt* and non-*Bt* treatments. Thus, our results agree with other field study conducted in USA that detected no significant effects of exposure to *Bt* pollen on colony performance (Rose *et al.*, 2007).

To sum up, the evolution between the groups of colonies was really similar (Fig. 1), without any significant difference along the experiment between groups, and the breeding mean ratio efficiency *Btk*/control increased throughout the experiment. So, the results of the effect of *Btk* aerial treatment (which is applied against processionary caterpillar in pine forest in Ibiza) on *A. mellifera* suggest that the *Btk* do not affect the brood development of honeybees. However, this is the first field assay conducted to assess the impact of aerial application on bees, so further field studies are required to ascertain a safe use of *Btk* in forest pest management.

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