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ORIGINAL ARTICLE

Farm-scale evaluation of the impact of Cry1Ab Bt maize on canopy nontarget arthropods: a 3-year study

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Abstract The cultivation of Cry1Ab-expressing genetically modified MON810 (Bt maize) has led to public concern in Europe, regarding its impact on nontarget arthropods (NTAs). We have assessed the potential effects of DKC 6451 YG (MON810) maize on canopy NTAs in a farm-scale study performed in Central Spain during 3 years. The study focused on hemipteran herbivores (leafhoppers and planthoppers) and hymenopteran parasitic wasps (mymarids) collected by yellow sticky traps, which accounted for 72% of the total number of insects studied. The dynamics and abundance of these groups varied among years, but no significant differences were found between Bt and non-Bt maize, indicating that Bt maize had no negative effect on these taxa. Nonetheless, the Cry1Ab toxin was detected in 2 different arthropods collected from Bt maize foliage, the cicadellids *Zyginidia scutellaris* and *Empoasca* spp. A retrospective power analysis on the arthropod abundance data for our field trials has determined that *Z. scutellaris* and the family Mymaridae have high capacity to detect differences between the Bt maize and its isogenic counterpart. The use of these canopy NTAs as surrogates for assessing environmental impacts of Bt maize is discussed.

Key words Cicadellidae; MON810; Mymaridae; risk assessment; surrogate species; *Zyginidia scutellaris*

Introduction

Maize expressing the insecticidal toxin Cry1Ab derived from *Bacillus thuringiensis* (Bt maize) has been commercialized in Spain since 1998 to control 2 major pests: the Mediterranean corn borer, *Sesamia nonagrioides* (Lefèbvre) (Lepidoptera: Noctuidae) and the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Farinós *et al.*, 2012; Crava *et al.*, 2013). Compa CB (Event 176) was the only variety grown until 2003, but it was gradually replaced by MON810 hybrids and finally withdrawn from the market in 2006. Since then, the adoption of MON810 has progressively increased to reach about 30% of the total maize area cultivated in Spain. Varieties derived from the event MON810 are the only genetically modified (GM) crop allowed for cultivation in the European Union (EU), with Spain accounting for 92% of the total EU Bt maize area (James, 2014; http://www.magrama.gob.es/es/estadistica/temas/estadisticas-agrarias/agricultura/esyrce/).

The use of Bt maize in European agriculture has raised public disquiet about the effects that these GM plants may have on nontarget arthropods (NTAs) present in the agroecosystems (EFSA, 2010a). Field studies conducted in Spain have focused on NTAs of different functional groups (herbivores, predators, parasitoids, and detritivores), but no detrimental effects of Bt maize on any of the main taxa have been reported (Ortego *et al.*, 2009; Albajes *et al.*, 2012; Comas *et al.*, 2014). Specifically, no negative effects on above plant and ground-dwelling predators were associated with the cultivation of Bt maize (Bt-176 and MON810) in farm-scale multiyear studies performed in different geographical areas (de la Poza *et al.*, 2005; Farinós *et al.*, 2008; Albajes *et al.*, 2012). Similarly, no adverse effects were reported for

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MON810 on the soil microarthropod decomposer community (Arias-Martín *et al.*, 2016), and for Bt-176 on herbivores (Lumbierres *et al.*, 2004; Pons *et al.*, 2005) and parasitoids (Pons & Stary, 2003). The data compiled from these field studies have been used for the identification of representative taxa to be used as surrogates for environmental monitoring purposes (EFSA, 2010b). Surrogate species selection is based on various criteria, such as the exposure to the toxin, its predictive power to detect differences between GM plants and its near-isogenic line and the species' ecological and economic relevance (Todd *et al.*, 2008; EFSA, 2010b; Romeis *et al.*, 2014). Accordingly, the following species or taxa have been proposed as surrogates for the main functional groups of NTAs in maize fields under Mediterranean agroecological conditions: Homoptera, particularly Cicadellidae, for herbivores, *Orius* spp. (Heteroptera: Anthocoridae) and Araneae for predators inhabiting the aerial part of the plants, Carabidae, Araneae, and Staphylinidae for epigeal predatory arthropods, Collembola and Acari for detritivores and the Mymaridae for parasitoids (Albajes *et al.*, 2012, 2013; Arias-Martín, 2015).

This study focuses on highly mobile and small arthropods inhabiting and feeding within the maize canopy (mainly leafhoppers, planthoppers and mymarids) (Baquero & Jordana, 2002; Dively, 2005; Rauschen *et al.*, 2008). Leafhoppers (Cicadellidae) and planthoppers (Delphacidae) possess mouthparts modified for piercing plant tissues and extracting their fluid contents (Tonkyn & Whitcomb, 1987). Besides mechanically injuring plant tissues, they are important vectors of pathogens in agricultural crops (Batlle *et al.*, 2000; Nickel, 2003). Among the species of leafhoppers inhabiting maize canopy, *Zyginidia scutellaris* (Herrich-Schäffer) (Hemiptera: Cicadellidae) has been previously reported as a key herbivore for measuring the impact of Bt maize (Rauschen *et al.*, 2008; Albajes *et al.*, 2013). Parasitoid fairyflies (Hymenoptera: Mymaridae) are egg parasitoids of a wide range of insect orders, such as Coleoptera, Psocoptera, Diptera, or Thysanoptera, but their most common hosts in maize fields are leafhoppers and planthoppers (Gauld & Bolton, 1988; Chiappini & Huber, 2008). In fact, some studies have shown that their emergence correlates with preceding peaks of abundance of cicadellids in the field (Huber, 1986; Albajes *et al.*, 2009). They have been documented as biological pest control agents (Krugner *et al.*, 2008; Loch, 2008), making them suitable candidates to be used in NTA risk assessment.

Canopy NTAs present in maize could be exposed to Cry1Ab toxin expressed in green tissues of the maize plant through different ways, depending on their feeding mode (Szekacs *et al.*, 2010). Herbivore arthropods could acquire the toxin by the ingestion of green plant material, as it has been observed in *Z. scutellaris*, *Tetranychus urticae* Koch (Acari: Tetranychidae), and *Trigonotylus* spp. Fieber (Hemiptera: Miridae) collected on Bt maize (Obrist *et al.*, 2006). Detritivores can be exposed to plant Cry insecticidal proteins by feeding on different plant residues like plant fluids, debris, pollen or dead leaves. This is the case of the springtail *Entomobrya* spp. Rondani (Collembola: Entomobryidae) (Arias-Martín *et al.*, 2016), which can be found in the aerial parts of plants at certain times of their life cycle. At the same time, canopy herbivores and detritivores can transfer the toxins to higher trophic levels, since they serve as food or host to predators and parasitoids commonly found in maize foliage. Thus, exposure to Cry toxins in the field has been reported in predators belonging to different taxa, such as *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), *Orius majusculus* (Reuter) (Hemiptera: Anthocoridae) or *Stethorus punctillum* Weise (Coleoptera: Coccinellidae) (Harwood *et al.*, 2005; Obrist *et al.*, 2006; Álvarez-Alfageme *et al.*, 2008). The exposure of parasitoids to the toxin has been only demonstrated under laboratory conditions in *Anagrus nilaparvatae* Pang et Wang (Hymenoptera: Mymaridae) when parasitizing eggs of *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) feeding on Bt rice (Gao *et al.*, 2010).

Attempts have been made to assess the effect of Bt maize on the canopy fauna in the EU. Multiyear field trials have shown no negative effects on planthoppers, leafhoppers and/or mymarids for Bt-176 in Spain (Pons *et al.*, 2005), and MON810 (Eckert *et al.*, 2006; Rauschen *et al.*, 2008) and MON88017 expressing Cry3Bb1 (Rauschen *et al.*, 2010) in Germany. However, to our knowledge analogous field trials have not been reported for MON810 varieties in Spain, despite being the only EU country where it has been cultivated continuously on a large scale since 1998. Here we report a 3-year farm-scale study conducted with MON810 in Central Spain to: (i) assess the exposure of field collected leafhoppers and planthoppers to the Cry1Ab toxin; (ii) evaluate its impact on the abundance of NTAs inhabiting the maize canopy; and (iii) examine, through a retrospective power analysis, representative canopy NTAs to be used as surrogate species.

Materials and methods

Study area and experimental design

The study was conducted during the years 2009, 2010, and 2011 using the same experimental set-up described in Arias-Martín *et al.* (2016). In brief, the size of the experimental maize field was 3.5 ha, and it was located

in San Fernando de Henares (Madrid, Spain). Temperature and rainfall in the study area were registered (see supplementary figure in Arias-Martín *et al.*, 2016). A randomized block design was used, involving 3 blocks and 2 treatments that were the maize varieties: transgenic maize plants (DKC 6451 YG, event MON810) expressing the Cry1Ab toxin (Bt maize) and its near-isogenic line DKC 6450 (non-Bt maize). The size of each plot was 0.5 ha (80 m × 60 m), with corridors of 3 m between them. Plots were arranged to hold the same treatments throughout the study. The crop was grown under irrigation without crop rotation and it was managed according to local agronomical practices, excluding the use of insecticides.

Cry1Ab protein

Cry1Ab toxin (78.9% purity) for the ELISA's standard curve was supplied by Dr. Juan Ferré (University of Valencia, Spain) who obtained the toxin from *Escherichia coli* cultures, strain XL1-blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F0 proAB lacIqZDM15 Tn10 (Tetr)]), transformed with the plasmid pBD140 (Bosch *et al.*, 1994), kindly donated by Dr. R.A. de Maagd (Plant Research International B.V., Wageningen, the Netherlands).

Exposure of field-collected canopy phytophagous arthropods to Cry1Ab

Samples of maize plant material and of canopy arthropods were taken from Bt and non-Bt maize plots 1 month after sowing (vegetative stages V6-V7) in 2011. Maize samples consisted of the fourth leaf taken from 5 different plants of both types of maize. Arthropods were collected by sweep entomological-net sampling (40 cm diameter, mesh size < 1 mm). The entomological net was swept across the maize leaves and the arthropods captured were immediately frozen using dry ice to avoid the metabolization and/or excretion of the toxin. In the laboratory, they were placed in Petri dishes over dry ice and examined using a Leica M125 stereomicroscope (Leica Microsystems S.A., Barcelona, Spain) for taxonomic identification. The specimens were transferred into 1.5 mL Eppendorf tubes and stored at -20 °C for Cry1Ab quantification by ELISA.

Levels of the Cry1Ab toxin were measured by double-antibody sandwich enzyme-linked immunosorbent assays (DAS ELISA), using the Agdia Bt-Cry1Ab/Cry1Ac Microtiter Plate Kit (Elkhart, IN, USA). Maize leaf samples (each plant was considered a replicate) homogenized with 0.3 mL phosphate buffered saline were diluted by a factor of 1:40. Arthropod samples consisted of 1 specimen of *Z. scutellaris* (15 replicates), 3 specimens of *Empoasca* spp. (2 replicates), 1 specimen of *Macrosteles* spp. (8 replicates), and 10 specimens of Delphacidae (3 replicates). *Zyginidia scutellaris* samples were homogenized in 0.5 mL phosphate buffer and the rest in 0.3 mL. Next all the samples were centrifuged for 5 min at 16 900 × *g*. After this, 100 μL of each sample was introduced independently into the ELISA plate, along with Cry1Ab standards, positive control and negative control, following the manufacturer's instructions. Standard curves for quantification were made using as calibrators different concentration solutions (0.125, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, and 6 ng/mL) of the purified Cry1Ab protein. The limit of detection (LOD) was calculated using the following equation: $\mathrm{LOD} = 3\sigma/S$, where σ is the standard deviation of 8 buffer-only controls and S is the slope of the calibration curve (ICH, 2005). The resulting LOD was 0.17 ng Cry1Ab/mL protein solution, so the toxin was considered as nondetected when values were below this number. Spectrophotometric measurements were conducted in a microtiter plate reader at a wavelength of 650 nm, using VersaMax^{TM} Microplate Reader (Molecular Devices Inc., Sunnyvale, CA, USA).

Effects of Bt maize on the abundance of canopy nontarget arthropods

Canopy NTAs were collected from the aerial parts of the Bt and non-Bt maize plants using yellow sticky traps of 100 mm × 210 mm (Bug-Scan® IVOG® -System, Biobest, Westelo, Belgium). Yellow sticky traps were placed in 1 maize plant (in 2009 and 2010) or in 2 plants separated by 20 m (in 2011), located in the middle of the central row of each plot. Yellow sticky traps were placed every year as follows: 1 month after sowing, 1 trap was fixed to a bamboo stick and placed at 50 cm above the ground. After 2 months, when the maize plant was tough and high enough, the trap was fixed to the plant. At this time, an additional trap was added at 150 cm height to cover the maize foliage and all the home range of the canopy NTAs. Traps were always faced at right angle to maize rows. After 3 d in the field, traps were removed, covered with cling film and stored in the refrigerator at 4 °C until they were examined. The sampling period covered different maize growth stages (from ~V6 to R6), lasting from June 12 to October 1 in 2009 (11 sampling dates), June 4 to October 1 in 2010 (11 sampling dates) and June 3 to September 22 in 2011 (15 sampling dates). Samplings were carried out weekly in June and July and every 2 weeks in August and September. The insects collected in the central grid of the trap (70 cm^2) were counted and

identified to family level and, when possible, to genus and/or species level, using a Leica M125 stereomicroscope (Leica Microsystems S.A., Barcelona, Spain).

Statistical analysis

A Generalized Estimating Equations model (GEE) was used to determine the effects of Bt maize cultivation on the dynamics and abundance of different groups of canopy NTAs. The *sampling week* of the 3 years was standardized at 17 weeks and they were considered as a repeated-measure factor, so that data taken at each growing-maize season could be compared among years. The factors *treatment* (Bt and non-Bt), *year* (2009, 2010, and 2011), and *block* were used as fixed factors and the interactions *treatment* × *year* and *treatment* × *block* were also analyzed. In addition, the factor *sampling week* nested within *year* was analyzed, since the number of sampling dates varied from year to year. Differences in abundance of specimens of the most abundant groups (Cicadellidae, *Z. scutellaris*, *Empoasca* spp., *Macrosteles* spp., Delphacidae, Mymaridae, *Anagrus* spp., *Gonatocerus* spp., and Trichogrammatidae) were analyzed. To normalize data, dependent variables were transformed with logarithm Ln($y + 1$). For all analyses, when a significant interaction between the factors *year* and *treatment* was found, data were analyzed pooling the data of the 3 years to retain the total available degrees of freedom and to improve the error estimates.

A retrospective power analysis was performed on the abundance from the different canopy NTAs groups to identify those with the highest capacity to detect effects of Bt maize. To this end, first we performed an analysis of variance (ANOVA) with the factors: *treatment* (Bt and non-Bt), *year* (2009, 2010, and 2011) and *block*. In this case, the factor *sampling week* was not considered and canopy NTAs abundances were expressed as mean yellow sticky trap values, that is, all sampling dates in each trap were pooled. The interactions *treatment* × *year* and *treatment* × *block* were also analyzed. With this type of design, *treatment* × *year* and *treatment* × *block* interactions were significant ($P < 0.05$) only in *Gonatocerus* spp., so it was decided not to consider these interactions to improve the power of the statistical analysis. Also, we performed an ANOVA, using the same dependent variables described above, but considering each year separately. To normalize data, dependent variables were transformed with logarithm Ln($y + 1$). Next, we employed the results obtained in both ANOVA analyses to determine if our field trials were capable of detecting the effect of Bt maize on canopy NTA abundances. If not, field trials may lack the ability to discriminate in the abundance of canopy NTAs between treatments and thus give false negatives, which are nonsignificant differences when effects actually exist (Perry *et al.*, 2009; Comas *et al.*, 2015). These effects are called detectable effect (d_c) and are usually expressed relative to the control mean abundance as a percentage. The d_c was calculated from the square root of mean squares of the residual error term from the ANOVA analyses of the model described above, and was determined for each canopy NTA each year separately (2009, 2010, and 2011) and pooling the data of the 3 years. Type I error was fixed at $\alpha = 0.05$ and the statistical power at 0.8 ($P = 1 - \beta$, being β the type II error and considered here as $= 0.2$). Values of d_c under 50% are considered as acceptable for detecting an effect on a field trial (Perry *et al.*, 2003) and the lower the d_c for a given taxon, the higher the detection capability.

GEE and ANOVA analyses were performed using SPSS software (IBM© SPSS© Statistics, Version 20, 2011). The JMP v.11.1 statistical package was used for all analyses and power calculations (SAS, Version 9.4). All the data of variability showed in the text refer to the standard errors of the means. A significance level of $P < 0.05$ was used for all tests.

Results

Composition of the maize canopy community

A total of 27 205 arthropods captured by yellow sticky traps were assessed over the 3 maize growing seasons at the Bt and non-Bt plots (Table 1). The 4 main groups were hemipteran herbivores from the Auchenorryncha assemblage (leafhoppers and planthoppers), hymenopteran parasitic wasps, dipterans, and thysanopterans, accounting for 89% of the total number of insects collected during the study. Among them, 2 groups stood out numerically from the rest: leafhoppers (Cicadellidae), which accounted for 45% of the total, and fairyflies (Mymaridae), representing 26% of the total arthropods collected. The ratio between these 2 principal groups varied with year; in 2009 the parasitic wasps were more abundant than leafhoppers and the contrary in 2010 and 2011. Leafhoppers were mainly represented by *Z. scutellaris* (90%), *Empoasca* spp. (5%), and *Macrosteles* spp. (4%). Delphacidae were frequent in samples of 2010 and 2011, but their densities never exceeded 3%. Similarly, among fairyflies, 92% belonged to the genus *Anagrus* spp., followed by *Gonatocerus* spp. (6%). Among parasitic wasps, we also found specimens of Trichogrammatidae (1%) and the superfamily Ichneumonoidea (Braconidae and Ichneumonidae) (<1%). Other canopy arthropods captured were dipterans from the family Chloropidae (10.8%)

Table 1 Main canopy NTAs found in Bt and non-Bt maize during 3 consecutive years. Data represent total percentages of individuals of each taxon collected by yellow sticky trap per type of maize and year.

Order	Suborder	Superfamily	Family	Proportion of specimens (%)					
				2009		2010		2011	
				Bt	non-Bt	Bt	non-Bt	Bt	non-Bt
Hemiptera	Auchenorrhyncha		Cicadellidae	33.64	36.09	60.95	62.96	42.31	46.94
			Delphacidae	0.19	0.25	1.98	2.84	1.79	1.62
	Heteroptera		Anthocoridae	0.37	0.55	0.13	0.20	0.35	0.27
Hymenoptera		Chalcidoidea	Mymaridae	42.93	38.14	14.86	15.92	18.78	18.84
			Thrichogrammatidae	0.56	0.78	0.13	0.78	3.84	1.52
			Other	7.00	8.23	2.59	3.10	7.30	7.62
		Ichneumonoidea	Braconidae	0.12	0.08	0.22	0.09	0.27	0.17
			Ichneumonidae	0.06	0.00	0.03	0.06	0.42	0.35
Diptera			Chloropidae	10.15	8.87	12.46	8.67	12.24	11.94
Thysanoptera				4.22	5.75	6.17	4.64	10.40	8.89
Coleoptera			Coccinellidae	0.35	0.58	0.22	0.12	1.67	1.16
			Staphylinidae	0.12	0.33	0.06	0.09	0.24	0.15
Neuroptera			Chrysopidae	0.25	0.33	0.16	0.35	0.20	0.19
Araneae				0.04	0.02	0.03	0.17	0.18	0.33

and Thysanoptera (6.9%). In a lesser amount, different predatory arthropods inhabiting the maize canopy were found (1.6% of the total), such as spiders (Araneae), green lacewings (Neuroptera: Chrysopidae), different ladybird beetles (*S. punctillum*, *Coccinella septempunctata* and *Propylea quatuordecimpunctata*) (Coleoptera: Coccinellidae), rove beetles (Coleoptera: Staphylinidae) and predatory bugs, *Orius* spp. (Heteroptera: Anthocoridae).

Exposure to Cry1Ab of field-collected canopy phytophagous arthropods

Four canopy phytophagous arthropods that were profusely collected by sweep netting were selected, according to their abundances in yellow sticky traps, for the analysis of exposure to Cry1Ab: *Z. scutellaris*, *Empoasca* spp., *Macrosteles* spp., and Delphacidae. Mean concentrations of 117.2 μg Cry1Ab/g of dry weight (SE 4.8) (Bt maize leaves), 4.6 μg Cry1Ab/g of dry weight (SE 0.8) (*Z. scutellaris*), and 0.9 μg Cry1Ab/g of dry weight (SE 0.2) (*Empoasca* spp.) were detected in samples collected from Bt maize field. Thus, Cry1Ab protein level was about 25 fold lower in *Z. scutellaris* and 130 fold lower in *Empoasca* spp. than in Bt plants. Toxin was not detected in the rest of herbivores analyzed (*Macrosteles* spp. and Delphacidae). No Cry1Ab toxin was detected in those plant samples from non-Bt plots and in specimens captured there.

Abundance and population dynamics of the principal canopy NTAs present in Bt and non-Bt maize

No differences were found between Bt and non-Bt plots in the abundance and dynamics of the different Auchenorryncha (cicadellids and delphacids) evaluated (Table 2). However, significant differences among years were found in these groups, except for *Macrosteles* spp. The most abundant cicadellid, *Z. scutellaris*, presented higher numbers in 2009 and 2010 than in 2011. This species displayed 2 peaks of abundance in June and July, respectively, and afterwards their densities diminished (Fig. 1). *Empoasca* spp. showed irregular dynamics depending on the year (Fig. 1). Dynamics of *Macrosteles* spp. and Delphacidae presented in most cases only 1 peak of abundance at the start of summer (June to July) (Fig. 1). Mymarid parasitic hymenopterans, mainly represented by *Anagrus* spp., showed similar numbers in Bt and non-Bt plots and presented significant differences among years, possibly related with the high abundance displayed in 2009 (Table 2). The dynamics of *Anagrus* spp. on non-Bt and Bt maize fields showed that this parasitoid presented a peak of abundance 3–4 month after maize sow (late July to August), depending on the year (Fig. 2). The number of *Gonatocerus* spp. and Trichogrammatidae collected in yellow-sticky traps were limited during the 3 years and both groups presented similar abundance and dynamics regardless the type of maize (Table 2; Fig. 2).

Table 2 Abundance of canopy NTAs collected by yellow sticky traps in Bt and non-Bt maize plots during 3 consecutive years.

Order	Group	Abundance per yellow sticky trap (mean ± SE)†						GEE analysis‡		
		2009		2010		2011		Year	Treatment	Year × Treatment
		Bt	non-Bt	Bt	non-Bt	Bt	non-Bt	Wald χ^2 (P value) (df = 2)	Wald χ^2 (P value) (df = 1)	Wald χ^2 (P value) (df = 2)
Hemiptera	Cicadellidae	32.68 ± 3.84	38.56 ± 4.91	37.39 ± 7.08	41.75 ± 5.39	16.06 ± 1.11	16.67 ± 1.06	128.44 (<0.00)*	1.53 (0.22)	3.18 (0.20)
	Z. scutellaris	31.14 ± 3.81	37.02 ± 4.85	34.71 ± 6.75	39.67 ± 5.26	13.35 ± 1.03	13.62 ± 0.96	171.39 (<0.00)*	1.49 (0.22)	3.14 (0.21)
	Empoasca spp.	0.38 ± 0.15	0.13 ± 0.06	1.18 ± 0.37	0.77 ± 0.15	1.32 ± 0.14	1.99 ± 0.21	§	§	7.09 (0.03)*
	Macrosteles spp.	0.90 ± 0.30	1.08 ± 0.35	1.04 ± 0.21	0.94 ± 0.19	1.17 ± 0.17	0.79 ± 0.12	5.28 (0.07)	0.56 (0.45)	4.51 (0.11)
	Delphacidae	0.18 ± 0.07	0.27 ± 0.09	1.65 ± 0.44	1.88 ± 0.52	0.68 ± 0.11	0.58 ± 0.10	91.25 (<0.00)*	0.22 (0.64)	3.91 (0.14)
Hymeno-ptera	Mymaridae	41.70 ± 6.87	40.75 ± 6.10	9.12 ± 1.21	10.56 ± 1.49	7.13 ± 0.56	6.69 ± 0.53	446.28 (<0.00)*	0.00 (0.99)	1.37 (0.50)
	Anagrus spp.	39.56 ± 6.76	37.15 ± 5.94	8.82 ± 1.19	10.12 ± 1.44	6.13 ± 0.54	5.88 ± 0.54	272.30 (<0.00)*	0.01 (0.92)	0.43 (0.81)
	Gonatocerus spp.	1.72 ± 0.32	3.27 ± 0.56	0.22 ± 0.07	0.29 ± 0.10	0.77 ± 0.10	0.55 ± 0.08	§	§	28.30 (<0.00)*
	Trichogrammatidae	0.54 ± 0.14	0.83 ± 0.18	0.08 ± 0.04	0.52 ± 0.15	1.46 ± 0.24	0.54 ± 0.08	§	§	21.91 (<0.00)*

*Significant differences ($P < 0.05$).

†The number of yellow sticky traps were 50 (2009, Bt plots), 48 (2009, non-Bt plots), 51 (2010, Bt plots), 52 (2010, non-Bt plots), 144 (2011, Bt plots), and 146 (2011, non-Bt plots).

‡Generalized Estimating Equations analysis. The results of the factors *block*, *sampling date* nested within *year* and the interaction *block* × *treatment* are not shown.

§Results of the statistical analysis for the factors *year* and *treatment* have been omitted in the table when their interaction was significant. In this case, data were reanalyzed pooling the data of the 3 years. No significant differences were detected between treatments in *Empoasca* spp. (Wald $\chi^2 = 1.12$, $P = 0.29$), *Gonatocerus* spp. (Wald $\chi^2 = 0.01$, $P = 0.94$), and Trichogrammatidae (Wald $\chi^2 = 2.03$, $P = 0.16$).

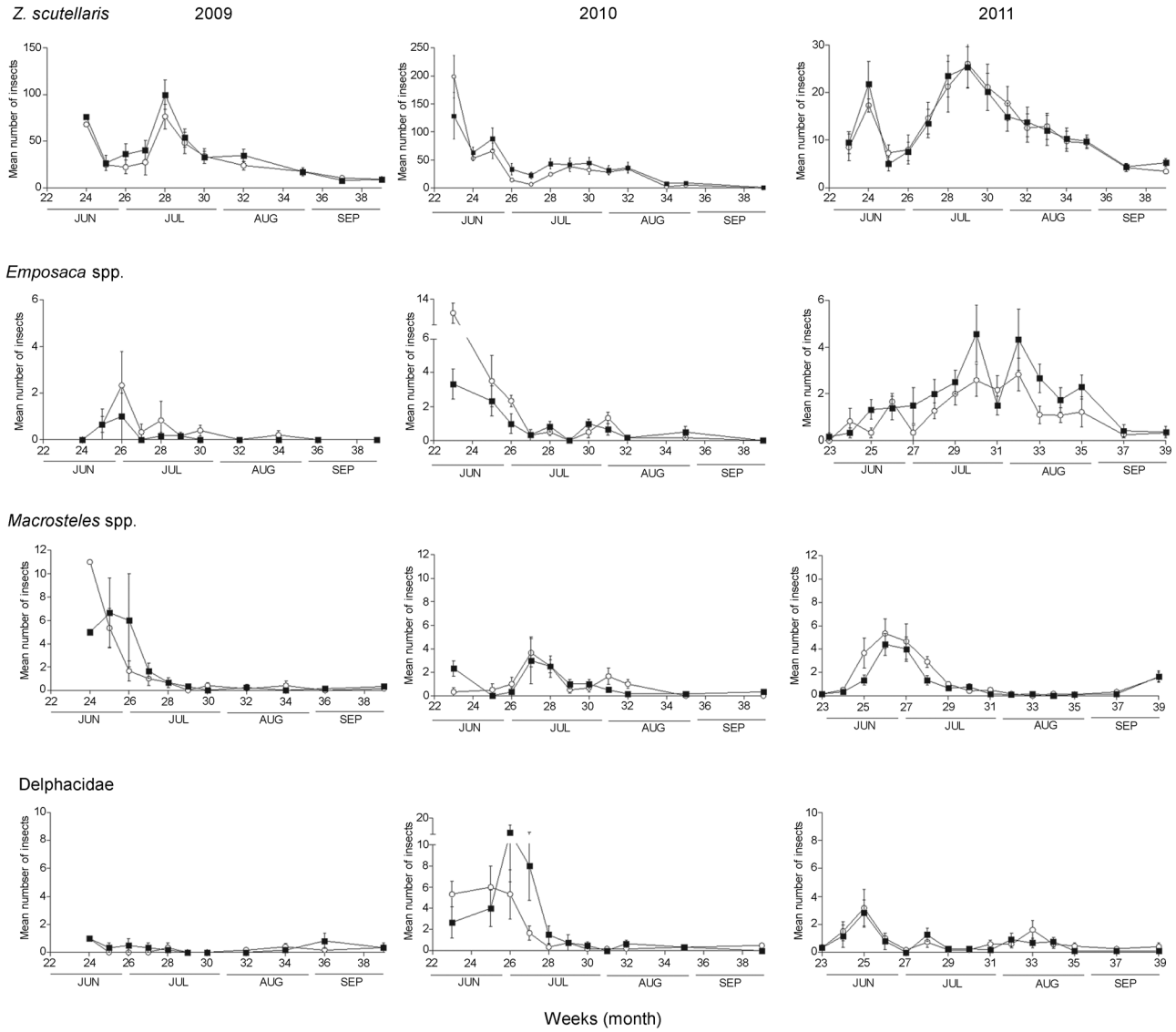

Fig. 1 Abundance of *Zyginidia scutellaris*, *Empoasca* spp., *Macrosteles* spp., and Delphacidae collected in Bt maize (—○—) and non-Bt maize (—■—) plots during the 3 years of study. Data are expressed as mean number (± SE) of specimens per yellow sticky trap.

Field trials' detectable effect (d_c) of the principal maize canopy NTAs collected in yellow sticky traps

The capacity of our field trials to detect differences in the abundance of NTAs between the Bt and the non-Bt maize fields varied depending on the group analyzed (Table 3). Some groups presented sufficient detection capacity (value of d_c under 50%) when years were considered separately, such as cicadellids (d_c ranged from 12% to 21%), *Z. scutellaris* (d_c ranged from 15% to 25%), mymarids (d_c ranged from 12% to 25%), and *Anagrus* spp. (d_c ranged from 18% to 31%). The rest of the groups presented variable d_c values that exceeded the 50% of the comparator mean value in at least 2 of the seasons, indicating a low capacity to detect an effect when actually exist. Interestingly, all the taxa evaluated showed higher capacity (lower d_c values) to detect differences between both types of maize when data of the 3 years were pooled. Specifically, cicadellids, *Z. scutellaris*, mymarids, *Anagrus* spp., and *Macrosteles* spp. displayed values of d_c under 50% (11%, 11%, 14%, 16%, and 45%, respectively).

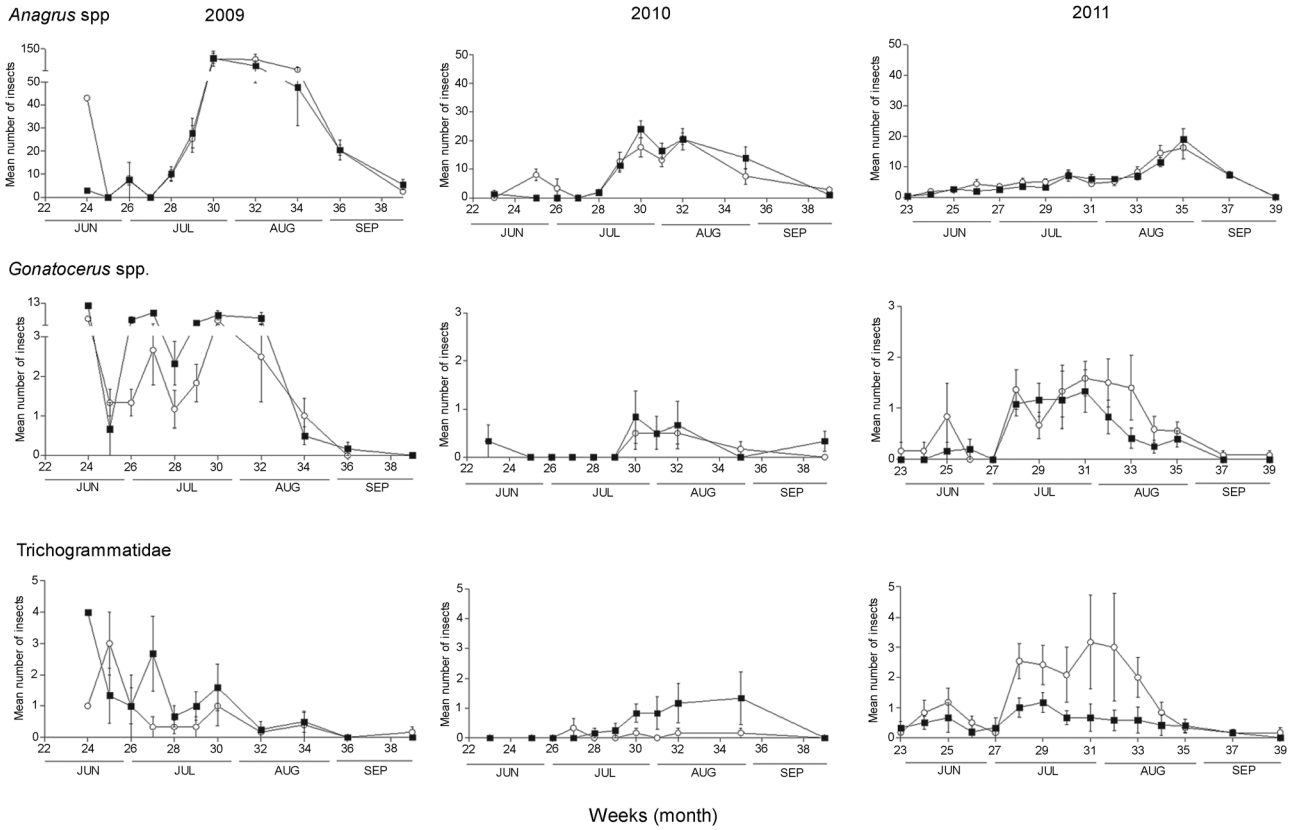

Fig. 2 Abundance of *Anagrus* spp., *Gonatocerus* spp., and Trichogrammatidae collected in Bt maize (—○—) and non-Bt maize (—■—) plots during the 3 years of study. Data are expressed as mean number (± SE) of specimens per yellow sticky trap.

Table 3 Detectable effect (d_c) of field trials for monitoring effects of Bt maize on canopy NTAs using yellow sticky traps. Results are expressed as percentages relative to the control mean abundance ($\alpha = 0.05$, $1 - \beta = 0.8$).

Order/group	d_c (%)			
	2009	2010	2011	2009–2011
Hemiptera				
Cicadellidae	12	18	21	11
Z. scutellaris	15	25	25	11
Empoasca spp.	>100	18	61	50
Macrosteles spp.	77	86	83	45
Delphacidae	>100	>100	98	65
Hymenoptera				
Mymaridae	25	12	25	14
Anagrus spp.	31	18	25	16
Gonatocerus spp.	31	95	>100	63
Trichogrammatidae	58	>100	>100	>100

Discussion

The assessment of NTAs exposure to Cry toxins is a central issue to determine the potential risk of GM crops under field conditions. In this study, we demonstrate that the toxin is present in 2 different arthropods inhabiting Bt maize fields, the cicadellids *Z. scutellaris* and *Empoasca* spp. at concentrations that correspond to 4% and 0.8% of the amount found in maize leaves. This is the first field report on the exposure of *Z. scutellaris* to Cry1Ab toxin from MON810-Bt plants, although the same toxin was previously detected at a concentration <0.2 μg Cry1Ab/g of dry weight in a pool of different stages of *Zyginidia* species collected on maize plants derived from event Bt-176 (Obrist *et al.*, 2006). Other studies have shown the passage of different toxins to cicadellids in similar ranges to our findings. Thus, the toxin Cry3Bb1 (event MON88017) was found in *Z. scutellaris* and *Empoasca pteridis* Göethe (Hemiptera: Cicadellidae) at rates of 1.2 and 0.8 μg Cry3Bb1/g of dry weight, respectively, representing 0.5% and 0.3% of the Cry3Bb1 toxin present in maize plant (Meissle & Romeis, 2009). Similarly, when

toxin Cry1Ac was measured on nymphs of *Cicadella viridis* L. (Homoptera: Cicadellidae) collected in Bt soybeans fields, the amount of toxin detected was 1.3 μg Cry1Ac/g of dry weight, that is the 3.5% of the toxin present in the plant (Yu *et al.*, 2014). We found no detectable levels of the toxin in the cicadellid *Macrosteles* spp. and in specimens of the family Delphacidae. The lack of detection in *Macrosteles* spp. might be related with the different types of feeding habits in leafhoppers (Tonkyn & Whitcomb, 1987). *Zyginidia scutellaris* and *Empoasca* spp. belong to the subfamily Typhlocybinae, characterized by feeding on the mesophyll cells (Backus, 1988). During the feeding process, they lacerate and ingest the content present in mesophyll and parenchyma cells, where the Cry1Ab toxin is produced at high levels (Abel & Adamczyk, 2004). However, xylem- and phloem-sap feeders as *Macrosteles* spp. are specialized in vascular tissue as their primary food source, where the Cry1Ab toxin has not been detected, or only as traces (Raps *et al.*, 2001; Rauschen *et al.*, 2010). In the case of the family Delphacidae, studies aimed to determine the level of exposure to Cry1Ab toxin at the species level would be necessary to contrast with our results, since the samples analyzed here were pools of species, with different feeding habits (phloem, xylem, or mesophyll cells) (Tonky & Whitcomb, 1987).

The potential effects of Bt maize on nontarget arthropod abundance were assessed in this 3-year field study throughout the maize growing season. We have found similar abundance of canopy NTAs in Bt and non-Bt maize, which indicates that the Cry1Ab toxin expressed in this GM crop has no effect on these arthropods. Moreover, all the groups evaluated presented similar dynamics each year, regardless the type of maize cultivated, whereas significant differences in abundance among years were common, suggesting that other factors rather than the use of GM maize were responsible for these population changes. Our results are consistent with other field studies that report no detrimental effects of maize varieties expressing Cry1Ab on the abundance of NTAs inhabiting the canopy of maize (Eckert *et al.*, 2006; Rose & Dively, 2007; Habuštová *et al.*, 2014); and more specifically, on the abundance of *Z. scutellaris* (Dively, 2005; Pons *et al.*, 2005; Rauschen *et al.,* 2008, 2010). It is worth pointing out the close bond between this species and maize crops, since it is its primary source of food when present (Nickel, 2003). Additionally, species of the genus *Anagrus* (Mymaridae) are among the most important parasitoids of *Z. scutellaris* in maize fields (Baquero, 1999). This relationship was evident in this study, where the peak presented by *Z. scutellaris* at the beginning of the maize season, coinciding with the initial stage of maize development (~V7–V9), was followed by the appearance and rise of *Anagrus* spp. a few weeks later. Comparable related dynamics were exhibited by cicadellids and mymarids collected by yellow sticky traps in field trials performed in northern Spain maize to assess risks of different GM crops on NTAs (Comas *et al.*, 2015).

The utility of field trials aimed to evaluate the effects of GM crops on NTAs depends on their capacity to detect an effect with a defined magnitude, with this effect usually fixed at 50% of NTA density or activity of the control (Perry *et al.*, 2003; Naranjo, 2005; EFSA, 2010b; Comas *et al.*, 2015). Besides, it is important to develop a species selection process adapted to each particular environment in which the GM crops will be used (Andow & Hilbeck, 2004; Romeis *et al.*, 2014). Based on field trials performed in northern Spain, the phytophagous species *Z. scutellaris* and parasitoid wasps of the Mymaridae were proposed as useful groups to evaluate the impact of GM maize crops on canopy NTAs (Albajes *et al.*, 2013). We have found that they were the most abundant and consistently captured taxa in our 3-year field study in Central Spain, and retrospective power analysis of our data showed that they presented the highest capacity among all sampled groups to detect differences between Bt and non-Bt maize (lowest d_c values). In addition, we have demonstrated that *Z. scutellaris* is exposed to the toxin Cry1Ab of MON810 maize, and it is the only cicadellid present throughout the entire maize growing period. These factors are crucial criteria to select *Z. scutellaris* as a surrogate species for environmental risk assessment. In the case of mymarids, whose exposure to the toxin in the field has not been evidenced, other criteria were taken into account for its selection, such as their abundance and ecological function in the maize agroecosystem. Most of mymarids collected belonged to the genus *Anagrus* spp., and this genus by itself showed high capacity to detect differences in our field trials. However, their taxonomic classification to the genus level is time-consuming and requires highly qualified personnel. Thus, the family Mymaridae is a better option to be used as a surrogate group for assessing indirect effects of Bt maize on parasitoids living within the maize canopy. The aggregation of different species within a taxon has been previously justified from an ecological perspective if only species with the same ecological functions are included (Comas *et al.*, 2013). Interestingly, similar values of d_c were obtained when the power analysis was performed at 2 levels (each year separately or pooling the data). Comparable results were found by Duan *et al.* (2006), who informed that the most abundant taxa would require few replicated blocks or years of study due to their high sample sizes, always on the condition that the variance of the data resulting

from the increase in the number of years does not counterbalance the increase in sample size. Likewise, Comas *et al.* (2015) concluded that the addition of seasons does not produce an increment of the capacity to detect differences in groups with high abundances. Nevertheless, we should not ignore that abundance of arthropod populations in the field are subject to many biotic and abiotic variables, such as the availability and quality of food, temperature, rainfall and the presence of natural enemies. Therefore, multiyear field studies assessing changes in the abundance of NTAs will provide more realistic information that could reveal ecological effects on arthropod communities that otherwise would be missed. In all other cases, the groups analyzed presented high capacity to detect differences only when data of the 3 years were pooled (as was the case of *Macrosteles* spp.) or in a particular year, demonstrating that they have poor effect-detecting capacities in our trials.

In summary, continuous cultivation of MON810 maize did not negatively affect the dynamics and abundance of the maize canopy NTAs studied in Central Spain, even though 2 cicadellids (*Z. scutellaris* and *Empoasca* spp.) were exposed to the Cry1Ab toxin. The leafhopper *Z. scutellaris* and parasitoids of the family Mymaridae proved to be the most suitable taxa to be used as surrogates of canopy NTAs for environmental risk assessment based on their frequency of occurrence throughout the study, exposure to the toxin, and capacity to detect changes caused by Bt maize. The same groups were identified as key taxa in field trials conducted in Northeastern Spain and in Germany to assess effects of different genetically engineered crops on NTAs, highlighting their value as surrogates under different agroecological conditions.

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Disclosure

The authors have no conflicts of interest; including involvement financial or otherwise, that might potentially bias the subject of this article.

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