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ORIGINAL PAPER

Refuge areas favor the presence of predators and herbivores in Bt soybean: a landscape perspective

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Abstract

Soybean plants that express various insecticidal proteins of the bacterium *Bacillus thuringiensis* have been widely adopted globally in many crop systems. This technology effectively controls the main defoliating pest species in most countries and reduces insecticide spray requirements. However, widespread use of Bt crops also generates high selection pressure against pest populations, leading to resistance concerns. Refuge areas are established to delay this phenomenon, but little is known about their other ecological functions. We evaluated the role of non-Bt soybean refuge areas regarding the abundance and richness of predator species in Bt soybean and non-Bt fields. For 2 years, herbivore and predatory arthropods were sampled in 28 soybean fields (RR/Bt) and their non-Bt soybean refuge areas (RR/noBt) in Uruguay, throughout the whole crop cycle. Landscape crop diversity (1 km radius) was characterized by its richness and evenness. Arthropod abundance and richness were analyzed using general linear mixed models. The abundance and richness of predators (Araneae, Coccinellidae, Heteroptera and Chrysopidae) found in Bt soybean were positively associated with the values recorded in refuge areas, independently of the diversity of the surrounding landscape. This relationship was not affected by changes in the distance between sampling points (within 800 m). The abundance of stink bugs and leaf-feeding caterpillars in Bt soybean was positively associated with refuge area values. Our results reinforce the importance of refuge areas, not only due to their role in resistance management, but also as preservation areas of beneficial fauna within a landscape approach to Integrated Pest Management in agroecosystems.

Keywords Insect-resistant GM crops · Landscape composition · Caterpillar pest · Predators · *Glycine max* · Insect-resistant management

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Key messages

- Predator abundance and richness did not differ between Bt soybean and non-Bt soybean refuge areas.
- Caterpillar abundance was higher in non-Bt soybean refuge areas than in Bt soybean.
- Bt soybean arthropod abundance and richness were positively correlated with non-Bt refuge areas.
- Landscape vegetation richness/evenness did not affect Bt soybean arthropods´ abundance/richness.
- We emphasize the role of non-Bt soybean refuges for the conservation of beneficial predators.

Introduction

Transgenic crops are planted in more than 185.1 million hectares globally (Singh et al. 2021) and they represent one of the primary strategies to improve crop productivity. Insect-resistant crops that express insecticidal proteins of the bacterium *Bacillus thuringiensis* (Bt) also allow for the reduction in chemical inputs (Dale 1999; Romeis et al. 2006; Qaim 2020). Since Bt crops have been commercially available since 1996, the area planted with Bt seeds worldwide has expanded rapidly (Tian et al. 2015), while numerous concerns about their adoption have been expressed among academics and across different segments of society. The potential impact of Bt crops on non-target organisms (Romeis et al. 2006; Yu et al. 2014; Marques et al. 2018; Luz et al. 2022) and the high selection pressure to develop resistant organisms (Gould 1998; Tabashnik et al. 2000, 2009) are among the most commonly raised issues. Regarding the potential impacts of Bt crops on non-target organisms, such as the impact on natural enemies, most of the scientific literature shows no negative effects (Comas et al. 2014; Romeis et al. 2019; Zhang et al. 2022). Moreover, many environmental and economic benefits have been reported due to their high specificity and the reduction in the use of insecticides (Carpenter 2010; Hutchison et al. 2010; Lu et al. 2012; Brookes and Barfoot 2018; Fleischer et al. 2021). However, the evolution of resistance to Bt proteins of the target pest species could diminish all these advantages (Tabashnik et al. 2013; Gassmann et al. 2014; Horikoshi et al. 2021a) and could compromise the efficacy of pest species control (Gould 1998; Hagenbucher et al. 2013).

Bt crops exert high selection pressure toward pest populations due to the high and continuous expression level of the toxin throughout the whole growing season of the crop (Tabashnik et al. 2013; Omoto et al. 2016; Santos-Amaya et al. 2016). There have been reported cases of insect field resistance in many crops and species (Van Rensburg 2007; Storer et al. 2010; Dhurua and Gujar 2011; Gassmann et al. 2011; Grimi et al. 2018; Horikoshi et al. 2021b). To mitigate this problem, since 1996, governments require farmers to plant a certain percentage of their transgenic crop acreage with non-Bt-producing cultivars (e.g., Macrae et al. 2005; Yano et al. 2016; Hutchison et al. 2010; Dourado et al. 2016). This management practice must concur with the adoption of Bt crops expressing high levels of the Bt protein, which should be sufficient to kill individuals heterozygous for a recessive resistance allele (Gould 1998). The objective of this area, called refuge, is to provide a percentage of the Bt's target pest population with the possibility of feeding on plants without toxins. Current resistance management modeling states that insect resistance may be delayed by agronomic practices if it is conferred by recessive alleles, but if it is not properly managed, resistance can develop anyway (Tabashnik 1994; Gould 1998). It is therefore expected that toxin-resistant moths that survive the transgenic Bt crop will have the ability to fly and mate with susceptible moths emerging from non-Bt refuges areas, diluting the frequencies of resistance alleles in the populations and producing heterozygous offspring which will once again succumb to the high-dose produced by Bt crops (Gould 2000; Papa 2022). Technical refuge recommendations depend on the crops deployed and vary by country. For example, in many countries of South America, non-Bt soybean is planted on at least 20% of the soybean acreage as a structured refuge, consisting of blocks or strips of non-Bt soybean, and located within 800 m of the Bt crop area (CTNBio 2010). Despite the widespread adoption of Bt crops in many countries, there is a rather low compliance with the proposed refuge strategy among growers (Fatoretto et al. 2017; Zuim et al. 2021). In many cases, non-structured or "natural" refuge areas are utilized to provide susceptible pest moths to mate with potentially resistant moths emerging from the Bt crop (Li et al. 2017). This is common in soybean in South America, where farmers have adopted variable proportions of non-Bt soybean growing near the Bt soybean as a refuge (when it fulfills the technical requirements), instead of sowing a structured refuge area.

Soybeans containing the stacked event MON 87701 × MON 89788 expressing the Cry1Ac insecticide Bt protein, and the 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) protein of *Agrobacterium* sp. have been available to farmers in Argentina, Brazil, Paraguay and Uruguay since 2013/14. In these countries, Bt soybean represented nearly 41% of the total soybean crop sown in the 2017/18 season (Brookes 2018) and reached 34.4 million ha in 2020/2021 (Bayer 2022). These events also confer glyphosate tolerance and control the most frequent soybean defoliating caterpillars *Anticarsia gemmatalis* (Hübner 1818) (Lepidoptera: Erebidae) and *Chrysodeixis includes* (Walker 1858) (Lepidoptera: Noctuidae), which are considered important pests across much of the Americas (Boernel et al. 1992; Abot et al. 1995; Bernardi et al. 2012; Borlotto et al. 2015; Perini et al. 2021). These events also control *Rachiplusia nu* (Guenée 1852) (Lepidoptera: Noctuidae) and *Crocidosema aporema* (Walsingham 1914) (Lepidoptera: Tortricidae) (Macrae et al. 2005), which are considered important lepidopteran pests in Uruguay and Argentina (Pastrana 2004; Barrionuevo et al. 2012; Perini et al. 2020). Recently, there is evidence of field-evolved resistance to MON 87701 × MON 89788 soybean by *R. nu and C. aporema* (Horikoshi et al. 2021b).

Beyond pest management, soybean crops support diverse taxa of parasitoids and generalist arthropod predators which can be effective in suppressing primary and secondary pests (Costamagna and Landis 2007; González et al. 2017, 2020; Samaranayake and Costamagna 2018). Within Bt crops, natural enemies also play another important role, as they can delay the development of resistant populations (Liu et al. 2014; Svobodová et al. 2017). Many of these natural enemies prefer to feed on eggs and first instars of caterpillars (de Sene and de Freitas Bueno 2019). In this sense, Bt transgenic crops have been modifying the composition of Lepidopteran fauna in soybean due to the reduction in the populations of dominant pest species, and likely affecting other arthropod species associated with the target species (Guedes et al. 2016, 2017).

Diverse hypotheses have been proposed to examine potential mechanisms by which Bt toxins could affect natural enemies, either through direct or indirect exposure (Andow et al. 2006; Romeis et al. 2006). However, very scarce literature has addressed the effects associated with the change in the abundance and composition of herbivore species in Bt soybean crops and the possible impact of these crops on natural enemies. In Bt soybean agroecosystems, which support less caterpillars, it is expected that the abundance of predators can be negatively affected by the lack of alternative preys. Under this scenario, the areas of refuge can harbor higher predator population levels, and these predators might provide additional ecosystem services (biological control, biodiversity and resistance management) due to a spillover from refuge areas onto Bt soybean or even neighboring crops, in addition to their objective of providing a pool of Bt-susceptible alleles in the population of the target pest (Tabashnik et al. 2009; Gassmann et al. 2014).

Environmental conditions are expected to differ between Bt crops and non-Bt crops (including refuge areas), determining the development of different insect communities between each other (Bianchi et al. 2006; Hagenbucher et al. 2013). Particularly in Uruguay, caterpillars (during the whole cycle) and stink bugs Heteroptera: Pentatomidae (during the reproductive stage) are considered the most important pest of soybean (Abbate et al. 2022a). In addition to those, during dry seasons, thrips and spider mites can also cause economic damages in the crop (Blanco et al. 2016; Perini et al. 2020). Since the Cry1Ac protein effectively controls the predominant caterpillar species, in seasons without water deficit, farmers usually do not apply insecticides during the vegetative stage in Bt fields. Based on economic thresholds (Bueno et al. 2011), in non-Bt fields and non-Bt soybean refuge areas, farmers apply insecticide to control caterpillars with relatively selective products (mainly triflumuron, chlorantraniliprole and methoxyfenozide) that generally result in mortality rates of less than 80% due to the various factors conditioning the spraying effectiveness under field conditions (Eckel et al. 1993; Follett et al. 1994). In addition, frequently in non-Bt soybean refuge fields of Uruguay, caterpillar generations do not reach the economic thresholds, providing permanent food sources associated with eggs and larvae of Lepidoptera for predators, which do not occur in Bt fields where the survival of caterpillars is nearly nil. In addition, two or three insecticide sprayings were needed in Bt and non-Bt refuge areas to control stink bugs during the reproductive stage.

Worldwide concerns have been raised about the widespread decline of arthropods (Sánchez-Bayo and Wyckhuys 2019), associated mainly with landscape simplification and agricultural intensification at the landscape level (Tscharntke et al. 2005; Seibold et al. 2019; van Klink et al. 2020). In recent years in Uruguay, there has been a 138% increase of agricultural land availability, primarily associated with increasing soybean acreage (MGAP-DIEA 2016). A recent report found that the proportion of this crop in the landscape negatively affects the abundance of predators in it (Abbate et al 2022b). Therefore, it is essential to identify the agricultural management practices and specific areas within the agroecosystems that contribute to the conservation or even to enhance the beneficial arthropod communities.

The purpose of the present study was to evaluate the role of non-Bt soybean refuge areas as a reservoir of predator and herbivore species to determine the following: (1) species abundance and richness; (2) whether these variables are correlated to those of Bt soybean; and (3) whether they are affected by the distance between non-Bt soybean refuge areas and Bt crops at a landscape level, as measured by the richness and evenness diversity indexes.

Materials and methods

Field study area

The study was performed during the 2018 and 2019 summer seasons in the western coastal region of Uruguay (Fig. 1, Table S1). The area belongs to the West Sediment Basin (Brazeiro et al. 2012), characterized by highly fertile soils

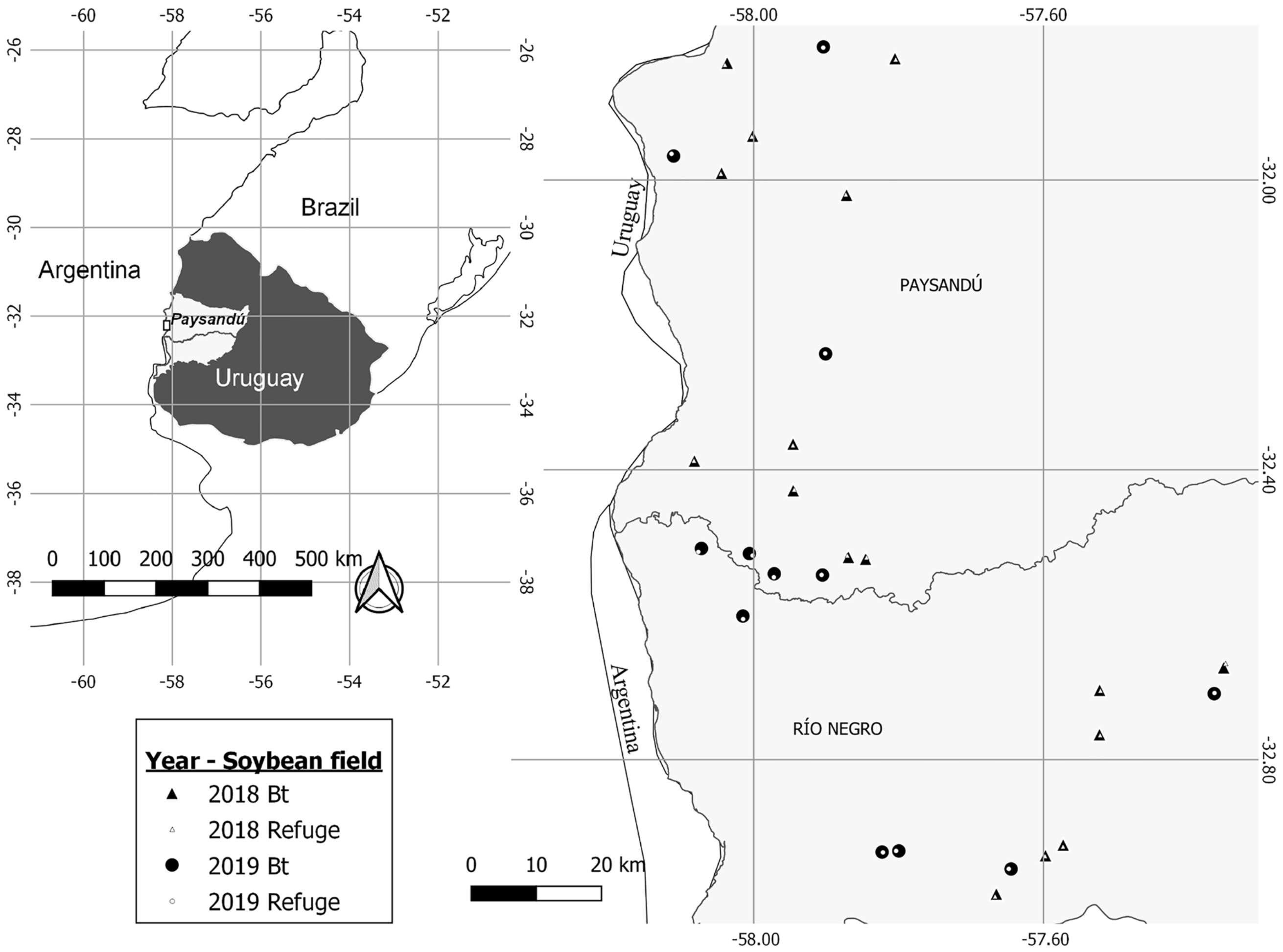

Fig. 1 Study region in the western coastal region of Uruguay (**a**); Bt soybean sampled fields and non-Bt soybean refuge areas (2018 and 2019) (**b**)

with an intensive use of land, representing nearly 78% of the total of the Uruguayan agricultural land dedicated to row crop production (Uruguay XXI 2016). The total crop area in this region increased during the past decade, primarily due to the sowing of soybean, advancing over native forest and grasslands areas (Tiscornia et al. 2014). Bt soybean represents nearly 29% of the total acreage of the crop sown in Uruguay (ISAAA 2018).

Arthropod sampling and landscape characterization

A total of 28 sites were sampled during a period of two soybean growing seasons ($n = 16$ in 2018 and $n = 12$ in 2019, Fig. 2, Table S1). Samples were collected using a sweep net on six sampling dates throughout the whole growing season. On each site, two geo-referenced points were marked, one located in a RR and Bt soybean crop (Intacta RR2 PRO®, event MON 87701 × MON 89788, hereinafter "Bt"), and the other point located in a non-structured non-Bt soybean refuge area, constituted by a RR and non-Bt soybean crop (Roundup Ready RR1®, hereinafter "non-Bt refuge"). This refuge crop area, which exceeded 20% of the Bt area, presented the same maturity cycle, sowing in a similar date (December), within a distance of 70–765 m from the sampled point of the Bt field and occupying a minimum of 20% of the total area of the Bt field, following the requirements for insect-resistance management programs (IRM). All the sampled fields (Bt and non-Bt refuge) corresponded to single soybean and none of them were irrigated. Fields sampled in 2018 were not repeated in 2019.

To estimate the landscape composition for each site, the vegetation cover composition was quantified using Google Earth satellite images for 2018 and 2019, followed up by ground truthing (e.g., Midega et al. 2014). The landscape variables identified in the field inspection were grouped into annual crops (soybean, maize and sorghum) and perennial crops (natural grasslands, native forest, commercial forest,

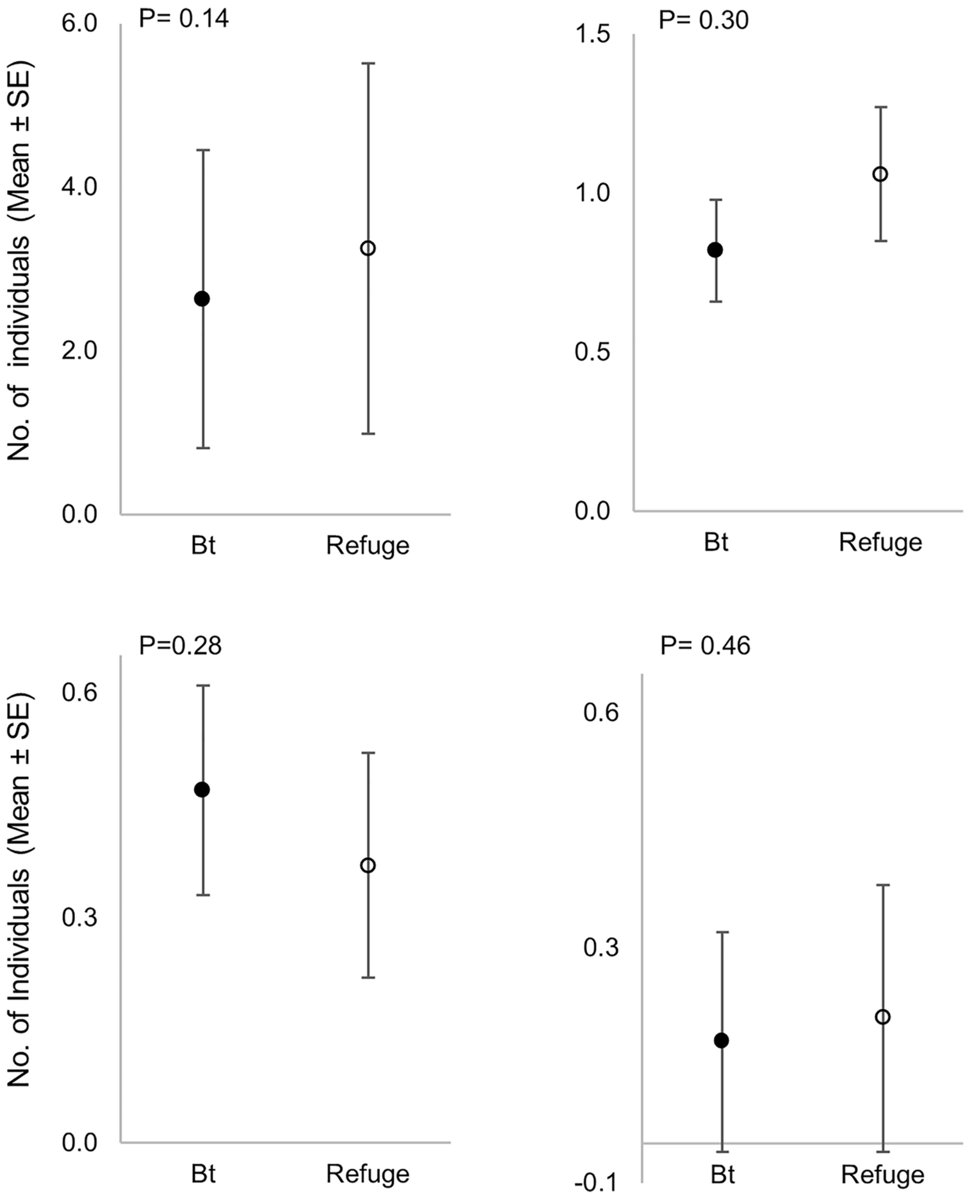

Fig. 2 Abundance of predatory arthropods: **a** Araneae, **b** Heteroptera, **c** Chrysopidae, **d** Coccinellidae, sampled in Bt soybean fields (solid circles) and non-Bt soybean refuges (empty circles). Values (mean ± standard error per 30 sweeps per sampled date) predicted by GLMMs. Significant differences between the technology's main effect, when $P < 0.05$

prairie with legumes and prairie without legumes). The QGIS software program (QGIS Development Team 2018) was used to delimit a 1 km radius around each site. Sampled fields were located in a gradient ranging from 30.6 to 90.8% of the landscape sown with annual summer crops in a 1 km radius buffer. Landscape vegetation cover diversity was characterized by crop richness and evenness, calculated based on the dominance concentration equation (Strong 2002), where the different landscape vegetation covers were expressed as a function of the proportional abundance and was calculated with the abdiv package (Bittinger 2020) of the R software (R Core team, 4.1.0 2021), (Table S1).

Each year, the arthropods (predators and herbivores) in 28 Bt soybean fields and adjacent non-Bt refuge areas were sampled on six sampling dates, every 3 weeks, during the whole crop cycle, from January to April (Table S1). A geo-referenced point was located in each field at 40 m from one margin (Woltz et al. 2012) to indicate the sampled universe.

Arthropod abundance was estimated with a sweep net (Ø 38 cm), following two transects of 15 m defined in the same direction of the crop rows, each one in opposite directions from the geo-referenced point. The arthropods were collected by sweeping 15 times in each transect. One sample was obtained for each transect and kept at − 18 °C until laboratory sorting. Both samples of the same site and date were added to be considered in the statistical analysis. Insects were identified at order, genus or species level depending on their state of conservation. Spiders were considered as a single taxon and not identified by family, genus or species. All the collected predators (immature and adults) were included, whereas only caterpillar and stink bug species (immature and adults) were identified among herbivores. For the statistical analysis, predator species were grouped into Araneae, Heteroptera, Chrysopidae, Coccinellidae, and herbivores into Noctuidae and Pentatomidae. To estimate predator richness, specimens of Araneae were classified in three categories according to their body size (< 0.5, 0.5–1, and > 1 cm).

Statistical analyses

Arthropod abundance and richness in Bt soybean and non-Bt soybean refuge areas

Predator and herbivore abundance and predator and stink bug richness in Bt soybean crops and non-Bt soybean refuge areas were evaluated with generalized linear mixed models that considered the following as fixed effects: technology (Bt and non-Bt refuge), phenological stage (vegetative and reproductive; Fehr and Caviness 1977), and the interaction between them (which was excluded when it was not significant). Year (2018 and 2019) and site nested in year were considered as random effects. Site nested in year contemplates the temporal correlations between each sampling date as a symmetric compound structure. The means of significant effects were separated using Tukey–Kramer's test ($P < 0.05$).

Association of arthropod abundance and richness between Bt soybean and non-Bt soybean refuge areas

Generalized linear mixed models were adjusted to study the relation of predator and herbivore abundance and predator and stink bug richness between Bt soybean and non-Bt soybean refuge areas. Year (2018 and 2019) and site nested in year were considered as random classification effects, while the phenological stage (vegetative and reproductive; Fehr and Caviness 1977) was included as a fixed classification effect. The regression variables included the following: abundance/richness of each arthropod group estimated in their respective non-Bt refuge areas, distance between the Bt and non-Bt refuge sampled sites, and richness and evenness of the landscape crop diversity (within a 1 km radius from the Bt point, Table S1).

All models were adjusted using a GLIMMIX procedure assuming a Poisson or negative binomial distribution with logarithm as link function of the statistical software SAS On Demand for Academics version 9.04 (SAS Institute Inc., Cary NC USA 2014), assuming a negative binomial (or Poisson with over-dispersion) error distributions and a log link function. Satterthwaite correction was used for degrees of freedom.

Results

Predator and herbivore abundance in Bt soybean and non-Bt soybean refuge areas

A total of 3621 arthropods were collected during the 2 years of the study: 1498 in Bt soybean fields and 2123 in non-Bt

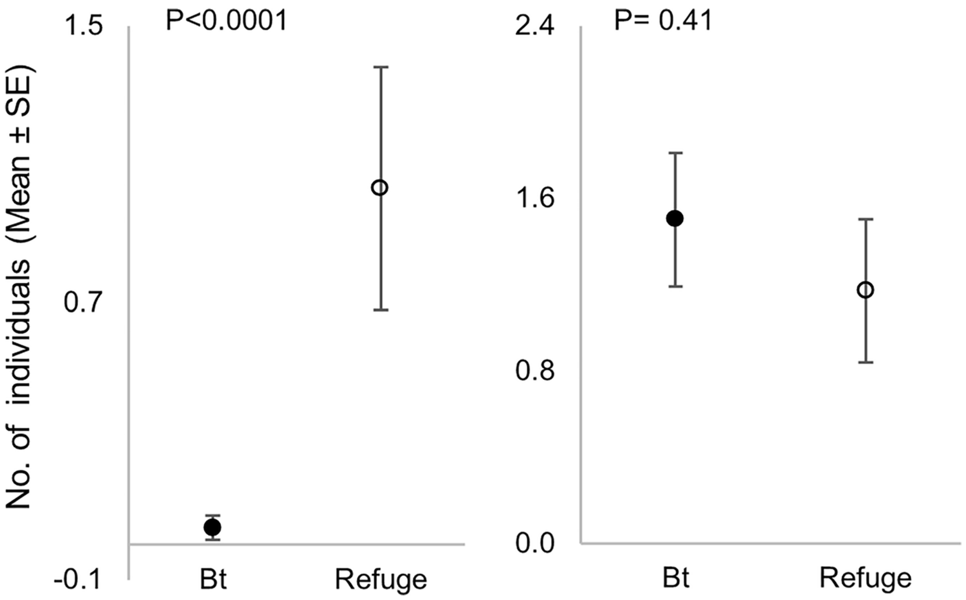

Fig. 3 Abundance of herbivore arthropods: **a** caterpillars, **b** stink bugs, sampled in Bt soybean fields (solid circles) and non-Bt soybean refuges (empty circles). Values (mean number of 30 times of sweep net per sampled date ± standard error) predicted by GLMMs. Significant differences between the technology's main effect, when $P < 0.05$

soybean refuge areas. Predators represented 75% and 61% of the total arthropods recorded in Bt and non-Bt soybean refuge areas, respectively. Among the herbivores sampled in Bt fields, stink bugs represented 23% and caterpillars only 2%. In non-Bt refuge areas, stink bugs and caterpillars accounted for 12% and 27%, respectively.

The abundance of the different groups of arthropod predators was not significantly different between Bt soybean fields and non-Bt soybean refuge areas (Fig. 2a–d, Tables S2). Among pests, the abundance of stink bugs did not differ between Bt and refuge soybean fields, whereas caterpillars were more abundant in non-Bt refuge areas (Fig. 3a, b, Table S3). Predator abundance was not affected by the phenological stage; caterpillars, on the other hand, were more abundant in the reproductive phase of the crop (Table S3). In Bt fields, stink bugs were more abundant during the reproductive stage, whereas in non-Bt refuge areas their abundance did not vary between crop stages (Fig. 3b, Table S3).

Specimens of the order Araneae were the most abundant among the total recorded predators (58.0% and 59.7% in Bt and non-Bt refuges, respectively). Heteroptera (Cimicomorpha) was the second most abundant group between predators (8.4% and 9.0% in Bt and non-Bt refuges, respectively), constituted mainly by *Orius* spp. [Anthocoridae], *Geocoris* spp. [Geocoridae], and *Nabis* spp. [Nabidae]. Coccinellidae predators represented 8.4% and 9.0% of all predators in Bt and non-Bt refuges, respectively, constituted mainly by *Eriopis connexa* (Germarar 1823), *Scymnus loewii* (Mulsant 1850) and *Hyperaspis festiva* (Mulsant 1850). Chrysopidae individuals were also recorded (9.2% in Bt and 5.9% in non-Bt refuges), mainly *Chrysoperla externa* (Hagen 1861). The few collected specimens of Carabidae, Mantispidae and Diptera predators were excluded from the analysis of abundance.

Piezodorus guildinii (Westwood 1837, Hemiptera: Pentatomidae) was the predominant stink bug recorded both years (81.3% in Bt and 82.1% in non-Bt refuge areas). *Dichelops furcatus* (Fabricius 1775) and *Edessa meditabunda* (Fabricius 1794) were the second and third species in abundance, respectively.

Among caterpillars, the velvetbean caterpillar (*Anticarsia gemmatalis,* Hübner 1818) was the most abundant species, followed by *Rachiplusia nu* (Guenée 1852) and *Chrysodeixis includens* (Walker 1857). *Spodoptera* species were the minority, mainly *Spodoptera cosmioides* (Walker 1858).

Predator and herbivore richness in Bt soybean and non-Bt soybean refuge areas

The richness of predators and stink bugs was not significantly different between Bt soybean fields and non-Bt soybean refuge areas (Fig. 4a, b, Table S4), but it varied between the phenological stages within stink bugs, being higher during the reproductive period of the crop (Tables S4).

Association of predator and herbivore abundance between Bt soybean and non-Bt soybean refuge areas

The increase in predator abundance in the non-Bt soybean refuge areas determined a significant increase in the abundance of all the predator groups evaluated in Bt soybean (Table 1). The regressions between the abundance of any predator group evaluated in Bt soybean fields and the landscape's indexes or the distance between both areas were not

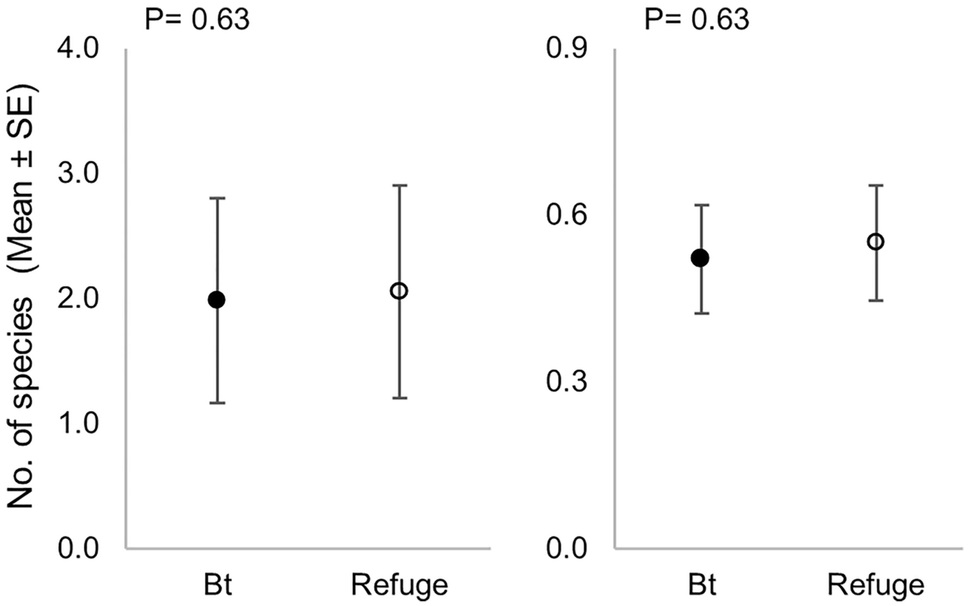

Fig. 4 Richness of predators (**a**) and stink bugs (**b**), sampled in Bt soybean fields (solid circles) and non-Bt soybean refuges (empty circles). Values (mean number of 30 times of sweep net per sampled date ± standard error) predicted by GLMMs. Significant differences between the technology's main effect, when $P < 0.05$

Table 1 Regression coefficients ($\hat{\beta}$), standard errors ($S_{\hat{\beta}}$) and *P* values of the independent variables included (predators abundance in refuge, landscape richness/evenness and distance between Bt and refuge) in the regression models used to predicted the abundance (mean no. per 30-sweeps) of the different groups of predators sampled in Bt soybean fields by generalized linear mixed models (GLMMs)

Group	Independent variable	Abundance of Predators in Bt soybean				
		$\hat{\beta}$	$S_{\hat{\beta}}$	df	t value	*P value*
Araneae	Abundance in refuge	0.0868	0.0131	88.2	6.6	**< 0.0001**
	Landscape richness	0.0249	0.0844	159.0	0.3	0.7681
	Landscape evenness	0.5084	0.7331	136.2	0.7	0.4891
	Distance Bt-refuge (m)	-0.0005	0.0005	159.0	−0.9	0.3682
Coccinellidae	Abundance in refuge	0.1546	0.0474	49.0	3.3	**0.0020**
	Landscape richness	−0.0927	0.3308	22.5	−0.3	0.7817
	Landscape evenness	−2.7070	2.3348	13.8	−1.2	0.2659
	Distance Bt-refuge (m)	0.0005	0.0018	17.1	0.3	0.7918
Heteroptera	Abundance in refuge	0.0927	0.0140	150.5	6.6	**< 0.0001**
	Landscape richness	0.1746	0.1677	30.2	1.0	0.3059
	Landscape evenness	0.5811	1.4628	22.0	0.4	0.6950
	Distance Bt-refuge (m)	−0.0006	0.0010	33.1	−0.6	0.5723
Chrysopidae	Abundance in refuge	0.27390	0.0889	155.5	3.1	**0.0024**
	Landscape richness	0.04444	0.1538	26.0	0.3	0.7749
	Landscape evenness	1.52980	1.5483	24.0	1.0	0.3330
	Distance Bt-refuge (m)	0.00003	0.0010	28.1	0.0	0.9745

Bold letters correspond to significative values (P value < 0.05)

Table 2 Regression coefficients ($\hat{\beta}$), standard errors ($S_{\hat{\beta}}$) and *P* values of the independent variables included (predators abundance in refuge, landscape richness/evenness and distance between Bt and refuge) in the regression models used to predicted the abundance (mean no. per 30-sweeps) of the different groups of herbivores sampled in Bt soybean fields by generalized linear mixed models (GLMMs)

Group	Independent variable	Abundance of Herbivores in Bt soybean				
		$\hat{\beta}$	$S_{\hat{\beta}}$	df	t value	*P value*
Caterpillars	Abundance in refuge	0.2261	0.0505	75.1	4.5	**< 0.0001**
	Landscape richness	0.2330	0.3041	27.9	0.8	0.4499
	Landscape evenness	−1.0345	3.0004	31.0	−0.3	0.7326
	Distance Bt-refuge (m)	−0.0018	0.0020	26.1	−0.9	0.384
Stink bugs	Abundance in refuge	0.0144	0.0049	120.1	2.9	**0.0040**
	Landscape richness	−0.0165	0.1627	27.1	−0.1	0.9199
	Landscape evenness	−0.0130	1.5454	22.7	0.0	0.9934
	Distance Bt-refuge (m)	0.0009	0.0009	16.7	1.0	0.3329

Bold letters correspond to significative values (P value < 0.05)

Table 3 Regression coefficients ($\hat{\beta}$), standard errors ($S_{\hat{\beta}}$) and *P* values of the independent variables included (predators abundance in refuge, landscape richness/evenness and distance between Bt and refuge) in the regression models used to predicted the richness (mean no. per 30-sweeps) of the different species of predators and stink bugs sampled in Bt soybean fields by generalized linear mixed models (GLMMs)

Group	Independent variable	Richness in Bt soybean				
		$\hat{\beta}$	$S_{\hat{\beta}}$	df	t value	*P value*
Predators	Richness in refuge	0.2142	0.0330	159.0	6.5	**< 0.0001**
	Landscape richness	0.0968	0.0639	26.5	1.5	0.1419
	Landscape evenness	0.0665	0.5678	20.1	0.1	0.9079
	Distance Bt-refuge (m)	0.0001	0.0004	25.3	0.4	0.7297
Stink bugs	Richness in refuge	0.3284	0.1128	159.0	2.9	**0.0041**
	Landscape richness	0.0619	0.0851	44.9	0.7	0.4704
	Landscape evenness	−1.2000	0.8236	159.0	−1.5	0.1471
	Distance Bt-refuge (m)	−0.0003	0.0006	159.0	−0.6	0.5471

Bold letters correspond to significative values (P value < 0.05) significant (Table 1). The phenological stage was not significant in the regression models of any arthropod groups with the exception of stink bugs (being more abundant in the reproductive stage, *P* value: 0.023).

The abundance of caterpillars in Bt soybean fields was significantly and positively dependent on their abundance in the refuge areas, while the associations with the landscape's crop diversity and the distance between Bt and refuge were not significant (Table 2). The abundance of stink bugs in Bt soybean fields was also significantly and positively dependent on their abundance in the refuges and the distance between the sampled points in the Bt fields and refuges (Table 2).

Association of predator and stink bug richness between Bt soybean and non-Bt soybean refuge areas

The richness of predators in Bt soybean fields was significantly and positively dependent on their richness in non-Bt refuges, but it did not significantly differ from the landscape's crop diversity nor from the richness in the distance between both areas (Table 3). The richness of stink bugs in the Bt soybean fields did not significantly differ from the regression variables studied (Table 3). The phenological stage was not significant in the regression models of any of the arthropod groups (Table S7).

Discussion

As expected, caterpillars were less abundant in Bt crops. However, and contrary to what we expected, the abundance of the most frequent predator groups (Araneae, Coccinellidae, Heteroptera and Chrysopidae) did not differ between Bt soybean and the non-Bt refuge areas. Regardless of this, our second hypothesis was confirmed, as predator abundance and richness in Bt soybean crops were positively associated with their abundance and richness in non-Bt soybean refuge areas, within a distance of 800 m. The same positive association was obtained for stink bug abundance. Contrary to our assumption, the landscape's richness or evenness had no effect on the studied variables for predators or herbivores, in concordance with other studies that affirm that different vegetation covers could diversely affect their abundance and richness (Landis et al. 2000; Samaranayake and Costamagna 2018; Madeira et al. 2019); thus, the effects could not be simply summarized in a single index.

Predator abundance and richness in Bt fields and non-Bt soybean refuge areas

The abundance of predators was not different between Bt fields and non-Bt refuge areas. This occurred despite the fact that Bt crops effectively host less available Lepidoptera's eggs and larvae, which are an important food source for the generalist predators (de Sene Pinto and de Freitas Bueno 2019). All predators evaluated are generalist and feed on a varied range of prey (Hurd 2008). In the case of Bt soybean, predators, when lacking a suitable prey, could feed on alternative food sources such as thrips and spider mites, considered secondary pests of soybean crops in South America. Higher predator abundance in Bt fields as compared to insecticide-treated non-Bt ones has been reported (Meissle et al. 2022). In the study region, to control caterpillars in non-Bt refuge areas, soybean is sprayed with relatively selective insecticides (mainly triflumuron, chlorantraniliprole and methoxyfenozide) that do not cause total mortality. These insecticide sprayings probably determined that predator abundance in those areas was not higher than in Bt fields. In addition, a number of eggs and larvae may survive and, together with secondary pests (thrips and spider mites), they could allow the predators to be abundant in the non-Bt refuges at the same level than in Bt fields. Previous studies conducted in the same region of Uruguay also determined the absence of differences in predator abundance between sprayed Bt and non-Bt soybean fields (Abbate et al. 2022b).

Predator richness was also not different between Bt fields and non-Bt refuge areas, suggesting that Crya1Ac does not negatively affect the community of predator arthropod fauna. Similar results were found in soybean at an experimental plot scale by Yu et al. (2014) and Marques et al. (2018), who did not observe differences of richness between sprayed and non-sprayed treatments of Bt and non-Bt soybean, associated with a quick recovery of the arthropod community diversity after spraying or a buffer capacity of the agroecosystems to the potential insecticide impact.

Herbivore abundance and stink bug richness in Bt fields and non-Bt refuge areas

Although the abundance of caterpillars was higher in the crop's reproductive stage than in the vegetative stage, a lower abundance of Noctuidae larvae was registered in Bt fields compared with non-Bt refuge areas in both crop stages, suggesting that the Bt technology remained effective for the control of the predominant caterpillar species of Uruguay. The high efficiency of Bt soybean was also reported in Brazil (Bernardi et al. 2012), although a resistance to Cry1Ac by *Rachiplusia nu* was recently observed (Horikoshi et al. 2021b), and control deficiencies were detected by farmers during the last crop season in some South American countries.

As for stink bugs, their abundance only differed between the different phenological stages in the Bt crops, but did not differ in non-Bt fields, which was probably associated with the presence of individuals belonging to *Edessa meditabunda* that can feed on soybean stems and leaves (Panizzi and Machado-Neto 1992), whereas the other species of the stink bug complex prefer reproductive structures (Bundy and McPherson 2000). In concordance with this, the richness of stink bugs was higher during the reproductive phase, when the beans and pods appear and the crop is colonized by species that prefer to feed on them (Panizzi et al. 2000). In the present study, the abundance and richness of stink bugs was not affected by the Bt protein, as expected, since they are not a target pest of this technology. However, an increase in mirid (Hemiptera: Miridae) populations has been registered in certain Bt crop systems as a result of the decrease in the application of broad-spectrum insecticides (Lu et al. 2010).

Association between predator and herbivore abundance and richness in Bt fields

Effect of non-Bt soybean refuge areas

The positive association observed in the abundance of generalist predators between Bt fields and their non-Bt refuges highlights the important role of the latter in different ecosystem services such as biological control, biodiversity and resistance management. In terms of their biological control function, our results indicate a strong relation between non-Bt refuge areas and predator abundance and richness in Bt soybean crops, suggesting that predators move between them. It is widely reported that predators move between different vegetation covers in search of alternative sources of food or protection areas from adversities such as the application of insecticides (Landis et al. 2000; Denys and Tscharntke 2002; Lu et al. 2012; Madeira et al. 2019). Several studies have demonstrated positive effects of insect pest consumption by generalist predators on soybean biomass and yield (Carter and Rypstra 1995; Costamagna et al. 2007), determining their role in biological control. As the number of individuals and species of arthropod predators in Bt soybean fields was directly associated with their values in non-Bt refuge areas, we also propose their contribution to maintaining diversity in agroecosystems, as it is well known that predators of all kinds play a critical role in maintaining biodiversity (Hurd 2008). In relation to the role of non-Bt refuges in the insect resistance management programs (IRM), in addition to providing susceptible Lepidoptera insects (Rausher 2001), the effects that they have on the abundance of predators should also contribute to IRM, as it was verified that natural enemies may delay insect resistance to Bt crops (Liu et al. 2014). Biological control, in conjunction with Bt crops, effectively suppresses the caterpillar pest population and decreases the development speed of resistant populations (Liu et al. 2014).

Recently, concerns have been raised about the widespread decline of arthropods (Sánchez-Bayo and Wyckhuys 2019); therefore, maintaining the diversity of entomofauna in agroecosystems should be a priority. The positive association of predator richness obtained between non-Bt refuges and Bt soybean crops showed the importance of complying with these areas to conserve species-rich assemblages of generalist predators and mitigate their decline in agroecosystems, mostly associated with habitat loss by landscape simplification and agricultural intensification (Seibold et al. 2019).

Landscape crop diversity effect

Despite the importance of soybean production in South America, little research has been designed to study the dynamics of non-target organisms in Bt crops at a landscape scale (Bueno et al. 2021). However, studies conducted in the same area of Uruguay determined that the proportion area of specific crops in the landscape presented different effects on generalist predators. The soybean cover area was negatively associated with the abundance of Araneae, Neuroptera and Hemiptera, whereas the cover areas of maize, natural grassland and commercial forest showed a positive association with some of those predators (Abbate et al. 2022b). In the present study, we observed that the diversity of the vegetation cover in the landscape surrounding Bt crops, measured through richness and evenness, did not affect the abundance and richness of predators, nor of herbivores. It is likely that, when these effects were combined in a single index, all associations counteracted each other and the effect could not be clearly detected, as natural enemy species may require quite specific resources at different times and spatial scales (Landis et al. 2005).

However, the species sampled have a limited range of hosts to feed on. In these cases, cover vegetation diversity per se is less relevant than how this diversity is constituted, as the identity of certain components is more important than the diversity among components (Barberi et al. 2010). Changes in arthropod communities was associated with changes in plant community composition rather than with overall plant richness (Haddad et al., 2001), and the presence of particular plant species had a greater effect on arthropods than plant taxonomic diversity (Koricheva et al. 2000).

In line with the thesis that habitat manipulations in agricultural landscapes could enhance biological control (Thies and Tscharntke 1999; Samaranayake and Costamagna 2019), here we propose that having non-Bt soybean refuge areas for Bt soybean would also foster this role. A high reduction of the agroecosystems' landscape heterogeneity has been taking place in South America during the last decades due to, among other things, the high adoption of Bt soybean and the set of intensive agronomic practices that produce high yields (Modernel et al. 2016; Brazeiro et al. 2020). This landscape homogenization causes adverse environmental conditions related with the reduction in the abundance and diversity of natural enemies and with the decrease in their efficiency as pest control agents (Landis et al. 2000). The preservation of non-Bt soybean refuge areas has greater relevance in the current simplified landscapes of South America.

Distance effect

We did not observe any effect in the abundance and richness of predators in Bt soybean fields associated with the distance from the non-Bt refuge areas, located within a distance of 800 m, as recommended by the IRM program. Our results suggest that, within the distance required to provide susceptible moth adults, non-Bt refuge areas could also provide positive associations of abundance and richness of predators with Bt soybean fields. In this sense, we hypothesize that non-Bt refuge areas could act as a source of predators for Bt crops and could enhance their populations within landscape agroecosystems through mobile arthropod dynamics (Topping et al. 2015). Nevertheless, it is expected that, in a larger range of distance, predator abundance associations between field and non-Bt refuges will become insignificant, as other variables may interfere in determining the dynamics of predator populations and communities in a range of ecological interactions. In the case of herbivores, as it was expected, caterpillar abundance was not affected by the distance between the range evaluated, affirming the IRM recommendations. On the contrary, stink bug abundance in Bt fields was positively associated with the distance from the non-Bt refuge area. These results may be associated with other results obtained in Brazil that related the adoption of transgenic soybean with the further increase in stink bug outbreaks (Guedes et al. 2017).

Conclusions

Our results enable us to conclude that the non-Bt soybean refuge areas, in addition to being a crucial tool for resistance management, play a key role in the conservation of the most common soybean predator groups, influencing their abundance and diversity in Bt soybean crops. We also found evidence that the non-Bt refuge areas might provide additional important ecosystem services such as biological control and the maintenance of biodiversity in the agroecosystems.

In order to assure these benefits, stakeholders in the soybean production chain should guarantee the compliance of non-Bt soybean refuge areas with all the structural requirements of distance, location and proportion area to match Insect Resistance Management Programs, the adoption of sampling, and economic thresholds to determine the need for control actions and the use of selective active ingredients. Maintaining a certain density of predatory and herbivore arthropods in non-Bt refuge areas is the key to provide the mentioned ecological functions (resistance management, biological control and biodiversity) that could only occur with the support of Integrated Pest Management at a landscape level.

Author contribution

SA, XP, OB and FM conceived and designed the research. SA collected the data. OB, FM and SA analyzed the data. SA, XP, NA and FM discussed the results. SA wrote the manuscript with the contributions of XP, NA and FM. All authors read and approved the manuscript.

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Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or vertebrates performed by any of the authors.

Informed consent The five authors of this manuscript agreed to submit it for publication in the Journal of Pest Science, and report that it has not been published or accepted for publication in another journal, and it is not under consideration for another journal.

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