UPEP
Unidad de Posgrados y Educación Permanente
FACULTAD DE AGRONOMÍA
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UNIVERSIDAD DE LA REPÚBLICA URUGUAY

Factores locales y de paisaje determinantes de la abundancia y riqueza de insectos plaga y artrópodos predadores en soja Bt y no Bt

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Doctora en Ciencias Agrarias
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Tesis aprobada por el tribunal integrado por el Dr. Eduardo Abreo, el Dr. Alejandro Costamagna y el Dr. Ezequiel González el 12 de Diciembre del 2022. Autora: Mag. Silvana Abbate. Director: Dr. Xavier Pons. Codirectora: Dra. Nora Altier.

Dedico este trabajo a mis hijos, Cami y Emi, porque no hubiese tenido sentido navegar si no fuera junto a ellos, a Andrés, por ser el timón del barco, y a mis padres, por haberme enseñado a elegir el destino al cual quiero llegar…

«Transformar el territorio en Paisaje es una mirada superior de Civilización»
EDUARDO MARTÍNEZ DE PISÓN

AGRADECIMIENTOS

En primer lugar, agradezco a la Universidad de la República por darme la oportunidad de formarme académicamente en el área que me gusta y de manera gratuita.

Agradezco muy especialmente a Nora y Xavier, tutores de excelente calidad humana y académica, por todo lo que aportaron a mi crecimiento profesional, siempre respetando y acompañando mi desarrollo personal, acompañándome y motivándome continuamente.

A los integrantes del tribunal, Alejandro Costamagna, Ezequiel González y Eduardo Abreo, por los aportes que realicen y su gran disposición para facilitar mi defensa en un momento del año lleno de compromisos. A Matilde Eizaguirre y a Antônio Panizzi, por todo lo que me enseñaron en mis pasantías.

A mis compañeros de Entomología, de Protección Vegetal y del GTI, quienes han sido fundamentales en este proceso: A Horacio, muy especialmente, por haber estado siempre dispuesto a enseñarme todo lo que no entendía, también a Adela, Agustina, Juana, Beatriz, Martín, Oswaldo, Sebastián, Luis, Tato y Javier por haber estado dispuestos siempre a darme una mano. A Líber Acosta y Graciela Carreño por el apoyo brindado en todo este período.

A Filipe Madeira, por su gran colaboración académica y motivacional durante todo el proceso de mi doctorado. A Oscar Bentancur, por su apoyo en el diseño y análisis de datos. A Tiago Lucini, por introducirme en las técnicas de EPG. A Ximena Cibils, por el tan enriquecedor intercambio que tenemos a diario y que tanto aporta a mi formación académica. A Diego Carpintero, Eugenia y Evelyn por su colaboración en la identificación de insectos. A Pablo Speranza quien me brindó un curso cuyos contenidos fueron fundamentales para la redacción de mis artículos.

Mi especial agradecimiento a todas las personas que participaron en los estudios de campo y laboratorio, sin los cuales no se hubiesen podido llevar a cabo: a mis dedicados tesistas de grado, Nancy, Valeria, Agustina, Micaela, Ana Paula, Giuliana y Luciano; a mis colegas, Agustina, Horacio, Luciana, Belén , Sebastián B., Nicolás y Cintia T., Winnona y a la gran cantidad de ingenieros agrónomos, asesores y

productores que amablemente me facilitaron sus chacras y me proporcionaron la información necesaria en todo momento.

A mis compañeros de la EEMAC, Enrique Cairus, David Gandolfo, Ana Manzi, Reynaldo Quintana, Julio Mosquerira, Carol Guilleminot y Patricia Choca, por facilitarme también parte de este camino. A Berenice Levin por su disposición a la corrección del formato de la tesis. A Gonzalo Peralta Attier por las correcciones de gramática inglesa, sintaxis, ortografía y estilo de los artículos.

A todos mis amigos, que hacen más llevadero cada fracaso y más valioso cada logro de la vida. Especialmente a Cintia, por transitar este gran desafío juntas.

Mi gran agradecimiento para mis hijos por el amor que me dan día a día y por ser mi mayor fuente de inspiración en cada paso que doy en la vida y a Andrés por darme siempre la mano en cada uno de nuestros caminos. Eterno agradecimiento a mis padres por su cariño infinito, por despertar mi interés por la agronomía, por los valores que me inculcaron y por demostrarme que los esfuerzos valen la pena. A Mage, por su una abuela incondicional, siempre dispuesta a ayudarnos. A mis hermanas, cuñados, sobrinas y sobrino, por ser parte de nuestras vidas y apoyarnos siempre.

Agradezco a todas las instituciones que aportaron de un modo u otro para que mi doctorado pueda ser llevado a cabo: EEMAC, CENUR Litoral Norte, FAGRO, UDL, INIA, EMBRAPA y ANII.

La realización de esta tesis fue posible gracias al apoyo de la Agencia Nacional de Investigación e Innovación ANII mediante el Fondo María Viñas (#FMV-2-2016-1-126142) y el Sistema Nacional de Becas de doctorados #POS_NAC_2011_1_3559 y de la Comisión Académica de Posgrado (CAP)-UDELAR mediante el Programa de Finalización de estudios de posgrado.

Los resultados obtenidos en esta tesis fueron difundidos en eventos nacionales e internacionales gracias a diversas fuentes de financiación de UDELAR e INIA. Los viajes de Xavier a Uruguay fueron posibles gracias al Programa de Vinculación con Científicos y Tecnólogos en el Exterior-ANII (CHA_1_2017_1_143339) y al Programa Movilidad e Intercambios Académicos: Profesores visitantes 2022, CSIC-UDELAR.

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RESUMEN

Los paisajes agropecuarios del bioma Pampas y Campos se modificaron considerablemente debido a la expansión de los cultivos anuales sobre zonas no cultivadas, la siembra de soja y la adopción de variedades resistentes a lepidópteros (tecnología Bt). Estos cambios en el paisaje tienen un gran impacto sobre los artrópodos predadores y fitófagos, poco estudiados en Uruguay. En esta tesis se evaluó: **a)** el efecto de la composición de la cobertura vegetal (factor del paisaje), de la tecnología Bt (variedades que expresan la proteína Cry1Ac) y de los márgenes de chacra (factores locales) en la abundancia de las principales especies plaga y predadoras del cultivo de soja**; b)** el rol de las áreas refugio (soja no Bt) en la determinación de la abundancia y riqueza de artrópodos predadores en soja Bt, y **c)** el efecto de la proteína Cry1Ac sobre la biología, reproducción y comportamiento alimenticio de *Piezodorus guildinii* (Hemiptera: Pentatomidae). En el litoral norte uruguayo, durante dos años, se muestrearon artrópodos predadores e insectos plaga en 90 chacras comerciales de soja no Bt, Bt y refugios. Se evaluaron los parámetros biológicos y reproductivos de *P. guildinii* alimentada con soja Bt y su posible efecto en el comportamiento alimenticio, utilizando la técnica de gráfico de penetración eléctrica. Los principales resultados obtenidos fueron: **a) i.** la soja Bt presentó menor abundancia de lepidópteros, pero esto no afectó la abundancia de predadores; **ii.** la abundancia de predadores en soja se relacionó negativamente con la proporción de dicho cultivo en el paisaje, pero positivamente con el campo natural y otros cultivos anuales, **iii.** la asociación entre la abundancia de predadores en los márgenes y dentro de la chacra de soja fue positiva; **b)** la abundancia y riqueza de predadores en soja Bt se asoció positivamente con los valores registrados en los refugios, evidenciando por primera vez el rol de estas áreas en la conservación de la fauna benéfica, y **c)** los parámetros biológicos, reproductivos y el hábito alimenticio de *P. guildinii* no se afectaron por la ingesta de la proteína Cry1Ac. Los resultados obtenidos aportan posibilidades de intensificación ecológica al diseño de los sistemas productivos nacionales y regionales, mediante un manejo de plagas abordado desde la óptica del paisaje.

Palabras clave: manejo de plagas en soja, control biológico natural, soja Bt

Local and landscape factors determining the abundance and richness of insect pests and arthropod predators in Bt and non-Bt soybean.

SUMMARY

Agricultural landscapes of the Pampas and Campos biome have changed considerably due to the expansion of annual crops over non-crop areas, soybean seeding and the adoption of lepidopteran-resistant varieties (Bt technology). These changes have a great impact on predatory and phytophagous arthropods, which have been scarcely studied in Uruguay. The following aspects were evaluated: (a) the effect of vegetation cover composition (landscape factor), Bt technology (varieties expressing Cry1Ac protein) and field margins (local factors), on the abundance of the main pests and predators of soybean; b) the role of refuge areas (non-Bt soybean) in determining the abundance and richness of arthropod predators in Bt soybean crops, and c) the effect of Cry1Ac protein on the biology, reproduction and feeding behavior of *Piezodorus guildinii* (Hemiptera: Pentatomidae). In the northern Uruguayan littoral, during two years, predators and pests were sampled in 90 commercial non-Bt and Bt soybean fields and refuge areas. Biological and reproductive parameters *of P. guildinii* fed with Bt soybean pods and their possible effect on feeding behavior were evaluated using the electrical penetration graph technique. The main results obtained were: a) i. Bt soybean presented lower abundance of defoliating lepidoptera, without affecting predator abundance; ii. Soybean predators' abundance was negatively related to the proportion of this crop in the landscape, but positively related to natural grassland and other crops; iii. Predators' abundance between margins and within soybean fields was positively associated; b) Bt predators' abundance and richness was positively associated with their values in refuge areas, evidencing for the first time their role in the conservation of beneficial fauna; c) biological and reproductive parameters and feeding behavior of *P. guildinii* were not affected by the ingestion of Cry1Ac protein expressed in Bt soybean pods. These results contribute to the possibilities of ecological intensification designs of national and regional production systems through pest management approached from a landscape perspective.

Keywords: pest management in soybean, natural biological control, Bt soybean

1. INTRODUCCIÓN

1.1. PLANTEO DEL PROBLEMA

La expansión e intensificación agrícola determinó incrementos en la productividad, lograda por la intervención del hombre mediante acciones realizadas desde el nivel molecular hasta el manejo del paisaje. En la actualidad, es abultada la evidencia que indica que los aumentos de los rendimientos se lograron a expensas, entre otras, de la degradación del ambiente y la alteración de servicios ecosistémicos como la biodiversidad y el control biológico (Tscharntke et al., 2021, Altieri y Nicholls, 2020, Tittonell et al., 2020, Habel et al., 2019, Garibaldi et al., 2016, Altieri y Nicholls, 2004, Thies et al., 2003, Tilman et al., 2002, Krebs et al., 1999, Thies y Tscharntke, 1999). La preocupación por los efectos colaterales de la agricultura sobre el ambiente se ha transformado en un fenómeno cultural global de este siglo, generando cuestionamientos cada vez más profundos. Las actuales interrogantes van más allá de la perpetuidad productiva de los cultivos y del límite de los establecimientos, ya que se ha evidenciado que los factores del paisaje no solo afectan la calidad ambiental, sino que además repercuten en el rendimiento logrado de los cultivos (González et al., 2020).

Para lograr una producción sustentable, resulta clave comprender en qué medida las actividades humanas alteran el ambiente y los servicios ecosistémicos e identificar prácticas de manejo que puedan contrarrestar los efectos negativos. Para entender el funcionamiento de un sistema y diagnosticar el riesgo asociado a un cambio, se debe estudiar cómo se acoplan los procesos implicados a distintas escalas (Janković et al., 2017, Levin, 1992). Muchas veces los fenómenos que operan a gran escala espacial y temporal generan restricciones que actúan en una escala menor (Madeira et al., 2022), y viceversa. La ecología del paisaje es una disciplina relativamente nueva que estudia el efecto de la heterogeneidad espacial y temporal sobre procesos ecológicos tales como la abundancia y distribución de los organismos (Garramuño, 2006). Este abordaje permite estudiar las propiedades emergentes que derivan de la integración entre distintas formas de vida ante el cambio en el paisaje y sus posibles efectos de mitigación en una red trófica (Odum, 1971), contribuyendo a entender la dinámica espacial y temporal de los insectos en los agroecosistemas (Kheirodin et al., 2020) y complementar los enfoques actuales de investigación y manejo de plagas (Garramuño, 2006). La generación de información en relación con los factores de paisaje y locales que afectan la dinámica de plagas y sus enemigos naturales resulta crucial para llevar adelante estrategias de intensificación ecológica (González et al., 2022, Bommarco et al., 2013), mediante las cuales se mejoran los indicadores económicos, ambientales y sociales de los sistemas agropecuarios (Ruggia et al., 2021).

1.1.1. Factores locales y del paisaje que afectan el manejo de plagas del cultivo de soja

En Uruguay y otros países integrantes del bioma Pampas y Campos (Garaycochea et al., 2020, Politis, 2008), la expansión agrícola determinó la simplificación del paisaje debido a la pérdida de heterogeneidad vegetal ocasionada por la remoción de hábitats naturales y praderas plurianuales, el aumento en el tamaño de chacras, el desmalezamiento de campos, el predominio de monocultivos, la aplicación de agroquímicos y a la adopción de cultivos transgénicos (Modernel et al., 2016, Ferraro y Benzi, 2015,Bianchi et al., 2006). Uruguay atravesó este proceso de agriculturización, incrementó la superficie de sistemas de agricultura continua en grandes extensiones y reemplazó el sistema mixto tradicional, en el cual los ciclos agrícolas rotan con períodos de pasturas (Gazzano et al., 2019, Ernst y Siri-Prieto, 2011). Entre los años 2000 y 2014, la agricultura de secano nacional se cuadriplicó y se intensificó, pasando de un cultivo por año a tres cada dos años. Este proceso llevó a que, en la actualidad, la diversidad de cultivos en el país se haya visto reducida al 75 % (Jobbágy y Aguiar, 2015). La expansión del cultivo de soja explica la mayor proporción de este crecimiento, representando más del 80 % de la superficie de cultivos estivales (MGAP-DIEA, 2019) y el 8 % del PBI nacional (Uruguay XXI, 2021).

Una de las consecuencias de la expansión de la soja en Uruguay ha sido el incremento en la cantidad de insecticidas empleados (Bruno, 2007) para contrarrestar el daño generado por los insectos fitófagos. Entre ellos, los lepidópteros defoliadores son considerados plaga primaria del cultivo (Bueno et al., 2021), debido a que provocan la pérdida de área foliar y, por ende, la reducción de la intercepción de luz y capacidad fotosintética, lo que acorta el período de llenado de granos (Board et al., 1997). Otras plagas primarias que afectan el cultivo durante la fase reproductiva son el complejo de chinches fitófagas (Bueno et al., 2021), entre las cuales se destaca *Piezodorus guildinii* por ser la especie más frecuente en el país (Abbate et al., 2022a) y con mayor capacidad de daño (Zerbino et al., 2016). Las chinches se alimentan del órgano a cosechar, lo que afecta directamente el rendimiento logrado (Panizzi et al., 2000).

En Uruguay, el complejo de lepidópteros defoliadores está constituido principalmente por *Anticarsia gemmatalis* y plusias (*Rachiplusia nu* y *Chrysodeixis includens*), las cuales son motivo de numerosas aplicaciones de insecticidas durante todo el ciclo (Blanco et al., 2016). El manejo de estas se ha basado exclusivamente en el control químico. A partir del 2012 se dispone de variedades de soja transgénica resistente a lepidópteros (Bt, *Bacillus turingiensis*), que contiene los eventos MON87701 y MON89788, otorgándole tolerancia al glifosato y resistencia a ciertos lepidópteros, esta última mediante la expresión de la endoproteína Cry1Ac. La adopción de variedades de soja Bt, en aproximadamente el 20 % de la superficie sembrada con este cultivo en el país, determina otra modificación en lo local (chacra) con posibles efectos a mayor escala.

En los agroecosistemas, el mantenimiento de predadores, parasitoides y entomopatógenos es de fundamental importancia como factor de equilibrio dinámico de las poblaciones de especies de artrópodos plagas (Losey y Vaughan, 2006, Degrande et al., 2002, Parra, 2000). Los enemigos naturales disminuyen la necesidad de intervención del hombre en el control de plagas (González et al., 2020, Costamagna y Landis 2007, Degrande et al., 2002) y mantienen hasta el 50% de las poblaciones de plagas por debajo de los niveles críticos en los que pueden reducir el rendimiento (Pimentel y Burgess, 2014). En soja, no logran mantener las poblaciones de plagas primarias por debajo de los umbrales de acción durante todo el ciclo del cultivo (Ribeiro, 2007), pero sí ejercen un rol importante en la primera etapa de colonización de la plaga retrasando el momento de la primera aplicación de insecticidas (Abbate et al., 2022a). Además, la acción de los biocontroladores resulta clave para mantener las plagas secundarias por debajo de los umbrales de daño económico tanto en soja como en pasturas y otros cultivos con los cuales se comparten ciertos enemigos naturales (Ribeiro, 2010).

Si bien la incorporación de cultivos Bt permite el control específico de determinadas plagas, disminuyen la necesidad de aplicación de insecticidas (Brookes y Barfoot, 2016, Shelton et al., 2002) y, por ende, reducen el impacto que estos presentan sobre los enemigos naturales (Romeis et al., 2019, Romeis et al., 2006), existen posibles riesgos que deben ser estudiados (Romeis et al., 2008, Groot y Dicke, 2002, Hails, 2002) en condiciones controladas y en el campo. Entre ellos se destacan los posibles efectos sobre organismos no diana (fitófagos o benéficos), debido a la toxicidad de la proteína adquirida directamente desde el vegetal o a través de la presa, o efectos indirectos asociados a la menor cantidad o la calidad de presas disponibles. Por otro lado, la reducción de las poblaciones de plagas objetivo de cultivos Bt, la disminución en el uso de insecticidas y el posible efecto sobre los controladores biológicos podrían favorecer el incremento de plagas no diana de esta tecnología, como, por ejemplo, las chinches fitófagas. En este sentido, la adopción generalizada de algodón Bt en diversas regiones del mundo implicó la disminución del uso de insecticidas de amplio espectro, lo que determinó indirectamente el aumento de la densidad de las poblaciones de chinches e incrementó los daños (Greene et al., 1999, Greene y Turnipseed, 1996) y las pérdidas (Olson et al., 2011) generadas por estas. Los efectos de la expresión de las proteínas Bt también podrían ocurrir de manera directa, mediante efectos subletales sobre especies no blanco, afectando su biología o comportamiento alimenticio, los cuales deberían ser evaluados tanto en condiciones de campo como en condiciones controladas. La técnica de gráficos de penetración eléctrica (EPG) incrementa las posibilidades de estudio de toxicidad de las proteínas Bt sobre insectos picosuctores, como es el caso de las chinches (Lucini y Panizzi 2017a, Schünemann et al., 2014).

Por otro lado, la rápida adopción mundial de los cultivos Bt y la constante expresión de los genes Cry en las plantas transgénicas determinó una fuerte presión de selección sobre las poblaciones de insectos plaga, lo que resultó en el desarrollo de poblaciones resistentes y en la pérdida de eficacia de control (Tabashnik et al., 2008, Alstad y Andow, 1995), ya documentada en diversos países del mundo (Horikoshi et al., 2021, Naik et al., 2018, Huang, et al., 2011). Con el objetivo de retrasar el desarrollo de resistencia en los insectos no diana, se han propuesto numerosas medidas dentro de los programas de manejo de resistencia de insectos plaga, MRI, (Head y Greenplate, 2012, Matten, 1998): rotación de toxinas, combinación de toxinas (genes apilados) y combinación de eventos con dosis altamente tóxicas y siembra de refugios: áreas de soja no Bt (Tabashnik et al., 2009, Brousseau et al., 1999). El uso de dosis alta/refugio es la estrategia ampliamente adoptada y fomentada tanto por las empresas privadas como por los organismos estatales. Si bien los programas de MRI tienen como objetivo retrasar la aparición de poblaciones resistentes, la realización de refugios cumpliría, además, un rol fundamental garantizando un mínimo de presas para mantener las poblaciones de biocontroladores que se alimentan de estas (White y Andow, 2003). En este sentido, hasta la fecha no existen trabajos que hayan abordado el rol ecológico de las áreas refugio asociado a la preservación de los enemigos naturales en los agroecosistemas con cultivos de soja.

La implementación de sistemas de agricultura continua y adopción de nuevas tecnologías no solo genera problemas ecológicos a escala de predio (local), sino también de paisaje (Emery et al., 2021, Roschewitz et al., 2005), pero las interacciones entre estos son aún parcialmente desconocidas (Jankovic´ et al., 2016). La intensificación de la agricultura afecta a diversos servicios ecosistémicos como el control biológico (Gonzalez et al., 2015, Perović et al., 2010), debido a que la simplificación del paisaje (entendida como el incremento del porcentaje de la superficie bajo agricultura) reduce la abundancia y diversidad de enemigos naturales (Samaranayake y Costamagna, 2018, Maisonhaute et al., 2017, Tscharntke et al., 2005), e incrementa el riesgo de surgimiento de plagas (Altieri y Nicholls, 2004). Los insectos fitófagos y sus enemigos naturales responden a la composición y disposición espacial de la cobertura del paisaje circundante (Woltz et al., 2012, Gardiner et al., 2009, Tscharntke et al., 2005). La complejidad del paisaje juega un rol de relevancia en la determinación de los niveles de insectos fitófagos en los campos agrícolas (Samaranayake y Costamagna, 2018, Tscharntke et al., 2005), ya que las áreas menos perturbadas proporcionan el *pool* de artrópodos benéficos que recolonizan el cultivo (Pompozzi et al., 2021, Lee et al*.,* 2001). Los márgenes de vegetación espontánea también resultan de gran interés en este sentido, debido a que incrementan la abundancia de enemigos naturales dentro del cultivo (Martin et al., 2019). En cultivos de soja inmersos en paisajes con mayor intensificación agrícola, las poblaciones de ciertas plagas son más abundantes (Maisonhaute et al., 2017, Noma et al., 2010) y el control natural por predadores generalistas es mayor en chacras ubicadas en áreas con cobertura más diversa (Gardiner et al., 2009). Sin embargo, hay excepciones en las que los hábitats naturales no determinan incrementos del control biológico natural de ciertas plagas (Kheirodin et al., 2022, Karp et al., 2018, Tscharntke et al., 2016).

Para lograr la sostenibilidad de los sistemas productivos uruguayos, se requiere generar conocimiento científico y desarrollo tecnológico local. En este sentido, los trabajos realizados en el marco de esta tesis pretenden brindar información de relevancia para contribuir en los programas sustentables de manejo de plagas a escala de paisaje. Para definir los objetivos de esta tesis se han tenido en cuenta:

— Los cambios en el escenario productivo del país y la región hacia sistemas de producción más intensivos que simplificaron los paisajes agrícolas-ganaderos.

— La importancia del cultivo de soja en dicho proceso y su relevancia en el PBI nacional actual y en la producción mundial de aceite y proteína.

— La relevancia de las chinches y lepidópteros defoliadores en el manejo sanitario de la soja, provocando pérdidas directas en la productividad del cultivo y ocasionando la aplicación reiterada de insecticidas para su control.

— El control biológico natural como importante servicio ecosistémico capaz de contrarrestar la densidad de insectos plaga.

— La adopción de variedades de soja Bt, representando en la actualidad, aproximadamente, el 20 % de la superficie total sembrada con dicho cultivo en Uruguay y, aproximadamente, el 40 % en la totalidad de los países del Mercosur.

1.2. HIPÓTESIS Y OBJETIVOS

1.2.1. Hipótesis

1) Existen factores locales y de paisaje que modifican la abundancia de los principales grupos de insectos plaga y artrópodos predadores del cultivo de soja:

1a) Los cultivos de soja inmersos en paisajes con predominio de cultivos anuales presentan mayor abundancia de plagas y menor abundancia de predadores.

1b) Los cultivos de soja Bt presentan menor abundancia de lepidópteros defoliadores, lo que afecta negativamente la abundancia y riqueza de predadores en comparación con las chacras de soja no Bt.

1c) La abundancia de predadores en la vegetación espontánea de los márgenes se asocia positivamente con la abundancia de estos dentro del cultivo.

2) Las áreas refugio de la tecnología Bt cumplen un rol funcional determinante de la abundancia y riqueza de las poblaciones de predadores en el cultivo de soja.

3) El comportamiento alimenticio, la biología y la reproducción de *P. guildinii* (especie plaga no diana de la tecnología Bt) no se ven afectados por el consumo de vainas de soja que expresan la proteína Cry1Ac; por lo tanto, el manejo de esta plaga no debería diferir entre cultivos de soja Bt y noBt.

1.2.2. Objetivo general

Estudiar la influencia de factores del paisaje (composición de la cobertura vegetal) y locales (tecnología Bt, las áreas refugio y los márgenes de chacra) en la abundancia y diversidad de insectos fitófagos y sus principales enemigos naturales en soja, con el fin de contribuir a un manejo sostenible de plagas.

1.2.3. Objetivos específicos

1) Evaluar el efecto de la composición de la cobertura vegetal (factor del paisaje) y la tecnología Bt y los márgenes de chacra (factores locales) en la abundancia de las principales especies plaga y predadoras del cultivo de soja.

2) Estudiar el rol de las áreas refugio en la determinación de la abundancia y riqueza de artrópodos predadores en cultivos de soja Bt.

3) Evaluar el efecto de la proteína Cry1Ac sobre la biología, reproducción y comportamiento alimenticio de *P. guildinii,* insecto plaga no diana, como factor local determinante de su abundancia en cultivos de soja Bt.

1.3. ESTRUCTURA GENERAL DE LA TESIS:

Consiste en tres artículos científicos que constituyen la estructura central de la tesis y un capítulo final de discusión general y conclusiones globales.

El artículo titulado «Association between landscape composition and the abundance of predator and herbivore arthropods in Bt and non-Bt soybean crops» fue publicado en la revista *Agriculture, Ecosystems and Environment* [https://doi.org/10.1016/j.agee.2022.108027] y sus autores fueron Silvana Abbate, Filipe Madeira, Horacio Silva, Nora Altier y Xavier Pons. Los resultados obtenidos en este trabajo abordan el primer objetivo específico y constituyen el segundo capítulo de esta tesis. En este trabajo evaluamos los efectos de la composición de la cobertura vegetal (1 km de radio) como variable de paisaje, y la adopción de la tecnología Bt como variable local, sobre la abundancia de las principales especies de insectos plaga y artrópodos predadores del cultivo de soja.

El artículo titulado «Refuge areas favor the presence of predators and herbivores in Bt soybean: A landscape perspective» fue aceptado en *Journal of Pest Science* [https://doi.org/10.1007/s10340-022-01580-7] y sus autores fueron Silvana Abbate, Filipe Madeira, Oscar Bentancur, Nora Altier y Xavier Pons. El objetivo de este trabajo fue evaluar el rol funcional de las áreas refugio en la abundancia y riqueza de los principales grupos de predadores de insectos plaga en la soja Bt y determinar si estas son afectadas por la diversidad de la cobertura vegetal circundante (1 km) y/o por la distancia entre el refugio y el cultivo Bt (800 metros máximo). Los resultados obtenidos en este trabajo se enmarcan en el segundo objetivo específico y corresponden al tercer capítulo de la tesis.

El tercer manuscrito titulado «Bt soybean expressing Cry1Ac does not affect the development, reproduction or feeding behavior of red-banded stink bug *Piezodorus guildinii* (Hemiptera: Pentatomidae)» tiene por autores a Silvana Abbate, Xavier Pons, Nora Altier, Tiago Lucini, Sebastián Bonansea, Oscar Bentancur, Agustina Armand Pilón, Horacio Silva, Giuliana De Souza, Luciano Irigoyen y Antônio Panizzi. Será enviado a la revista Neotropical Entomology a la brevedad. En el marco de este trabajo se aborda el tercer objetivo específico y constituye el cuarto capítulo de la tesis.

2. ASSOCIATION BETWEEN LANDSCAPE COMPOSITION AND THE ABUNDANCE OF PREDATOR AND HERBIVORE ARTHROPODS IN BT AND NON-BT SOYBEAN CROPS

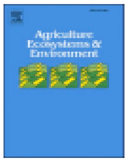
Abbate S, Madeira F, Silva H, Altier N, Pons X.2022. Agriculture, Ecosystems & Environment, 336: 108027. https://doi.org/10.1016/j.agee.2022.108027

Agriculture, Ecosystems and Environment 336 (2022) 108027

Contents lists available at ScienceDirect

Agriculture, Ecosystems and Environment

journal homepage: www.elsevier.com/locate/agee



Association between landscape composition and the abundance of predator and herbivore arthropods in Bt and non-Bt soybean crops

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ARTICLE INFO

Keywords:
Field margin
Natural grassland
Forest
Perennial crops
Annual crops
Stink bugs
Caterpillars
Spiders

ABSTRACT

Cropland expansion has been occurring over the last century, especially in South America, displacing non-crop areas mainly with soybean. Changes at a landscape level were followed by changes at a local scale, such as the adoption of insect-resistant transgenic crops. The homogenization of agroecosystem composition and agronomic practices have a great impact on arthropod predators and herbivore insects, which has not been quantified to a great extent in South America. We evaluate the effects of cover composition (1 km radii) as a landscape variable, and Bt adoption as a local variable on the quantity of the main predators and pests in soybean fields in Uruguay. Over a two-year period, we sampled 60 soybean fields with sweep net and beating sheet. We found that Bt and non-Bt soybean fields did not differ in the number of predators and stink bugs, while Bt fields were associated with less caterpillars' abundance. The proportion area of soybean negatively affected the quantity of some predators in this crop. In contrast, the landscape cover with natural grassland, commercial forest and maize positively affected some of these beneficial organisms. The number of stink bugs in soybean was highly affected by the surrounding cover composition. Arthropod responses depend on soybean's phenological period. We demonstrate the effects of the agriculturalization process on soybean predators and pests, suggesting that Bt adoption does not affect predators and each vegetation cover impacts them differentially. Different non-crop landscape cover and the abundance of predators in the field margin are positively associated with the abundance of predators in soybean fields.

1. Introduction

Human intervention has generated cropland expansion and intensification through changes at different scales, from the molecular to the landscape levels. Temperate grasslands have been the terrestrial ecosystem most affected by agricultural expansion (Henwood, 2010; Brazeiro et al., 2020). In South America, more than 15% of the natural grassland area of the Río de la Plata region (28–38°S and 47–67°W) was lost and fragmented in the last three decades (Paruelo et al., 2006). This process occurred mainly due to the expansion of genetically modified soybean (*Glycine max* L.) production (Jobbágy et al., 2006; Paruelo et al., 2006; Baldi and Paruelo, 2008; Modernel et al., 2016), which replaced natural vegetation, pastures and other croplands (Graesser et al., 2018; Zalles et al., 2019).

Soybean provides the largest source of vegetable protein in the world (Grassini et al., 2021). South America contributes with 48.5% of the

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https://doi.org/10.1016/j.agee.2022.108027
Received 23 December 2021; Received in revised form 3 April 2022; Accepted 13 May 2022

world's soybean production (Food and Agriculture Organization of the United Nations, 2019), being the region with the fastest growing area cultivated with this crop during the last two decades. In Uruguay, the government's national production targets of soybean and exotic forestry for 2030 (Office of Planning and Budget from Uruguay-OPP, 2009) would imply the loss of almost half (48%) of the natural habitat by that year (Brazeiro et al., 2020).

Soybean crops in the Rio de la Plata region present a wide diversity of herbivore insects but only a few species are considered primary pests. Those species are leaf-feeding caterpillars (Lepidoptera: Noctuoidea) and stink bugs (Hemiptera: Pentatomidae) (Bueno et al., 2021). Lepidopteran larvae consume leaves throughout the whole crop cycle, whereas stink bugs and some larvae of Noctuoidea feed on pods and seeds, causing significant yield losses (Panizzi et al., 2000; Bortolotto et al., 2015). A complex of generalist arthropod predators of many taxa is relatively common in soybean (spiders, hemipteran, coccinellid beetles) and can be effective suppressants of primary and secondary pests (Costamagna and Landis, 2007; González et al., 2017, 2020; Samaranayake and Costamagna, 2018).

Since 1996, glyphosate tolerant genotypes of soybean has been commercially available in Rio de la Plata region, carrying the event MON 89788 (cp4 epsps gene of *Agrobacterium* sp.; Roundup Ready RR1®). In 2012, Bt soybean Intacta RR2 PRO® was also approved, combining the events MON 89788, expressed in cell organelles different than RR1® (Justiniano et al., 2014), with MON 87701, expressing the Cry1Ac *Bacillus thuringiensis* (Bt) protein. Bt soybean conferred resistance to the more frequent lepidopteran pests in Brazil, Argentina, Paraguay and Uruguay, during the 2017/2018 crop season. Bt soybean represented 41% of the total acreage of this crop sown in these countries (Bueno et al., 2021). After four years of its adoption, a reduction of 7.4 million kg of insecticide active ingredients (6% of the total insecticide used in soybean) has been estimated (Brookes and Barfoot, 2018). Despite the savings in chemical control and the high specificity of Bt toxins, Bt plants could cause indirect effects due to a reduction in the number or quality of Bt target pests that natural enemies prey on (Tian et al., 2014). Changes in the status of herbivore pests could occur due to the absence of significant damage by the target pests (Wolfenbarger et al., 2008; Naranjo, 2009; Lu et al., 2010; Eisenring et al., 2019). Although there is extensive information on the direct and indirect effects of Bt toxins expressed in genetically modified crops (Comas et al., 2014; Tian et al., 2014; Romeis et al., 2019), very few studies address the effects at a field scale and the interaction with landscape variables (Wu et al., 2008; Lu et al., 2012).

Transgenic soybean (herbicide and insecticide resistant) currently exceeds 96% of the total soybean sown in all South American countries (International Service for the Acquisition of Agri-biotech Applications, 2018) facilitating the expansion and intensification of agriculture in the region, by the increase in field size and in the frequency of weed control resulting in the simplification of the landscape (Bianchi et al., 2006). Simplified landscapes are associated with lower biodiversity, which can affect ecosystem services, including biological control, and result in increased pest pressure and the emergence of new pests (Altieri and Nicholls, 2004; Venette, 2015; Balmford et al., 2018). The effects of converting greater proportions of the landscape to agriculture interact with the approach to pest management at a local scale (e.g., the technology used, chemical control). As an example, the expansion of annual summer crops (at a landscape level) and the replacement of crop-grazing rotations with continuous crop systems (at a local scale) benefit insect pests in grain sorghum in Uruguay (Emery et al., 2021).

In simplified landscapes, the ecological role of non-crop areas acquires greater importance as a source of natural enemies, providing alternative food sources, refuges and overwintering sites (Landis et al., 2000; Roschewitz et al., 2005; Thies et al., 2005). In that aspect, the spontaneous vegetation of field margins, natural grassland and native or commercial forests represent the least disturbed areas of South American agroecosystems. Many studies addressed the functional role of a natural habitat as a source of natural enemies, but only a few studied the importance of crop and pasture areas with a landscape approach (Madeira et al., 2021).

Although the cover and proximity of non-crop areas generally benefit the abundance of natural enemies (González et al., 2015), this does not necessarily lead to an increase in biological control (Karp et al., 2018), and in some cases pests also benefit from those areas (Tscharntke et al., 2016). However, studies performed around 1.5 km of soybean fields found a positively effect of diverse landscape composition in soybean aphid biocontrol by natural enemies (Gardiner et al., 2009). At a landscape level, native forest provides an abundance of natural enemies that contributes to the biological control of stink bugs in soybean crops (González et al., 2017), while natural vegetation areas could promote temporal stability for natural enemies by attenuating the generalized decline of their population during soybean senescence (González et al., 2016). Therefore, the study of the temporal variation in the natural enemies' response to landscape complexity would allow for a better understanding of the landscape effects (Chaplin-Kramer et al., 2013). Despite the important agriculturalization process that occurred in Uruguay and other South American countries, the approaches to determine the effects of landscape simplification on beneficial or pest arthropods and ecological services are scarce.

For a better understanding of the impact of landscape and local variables to maximize ecosystem services from conservation biological control in agroecosystems, the aim of the present work was to evaluate the effects of landscape composition and field local variables, especially Bt soybean adoption, on the abundance of predators and pests in soybean fields. We hypothesized the following:

1. Bt soybean fields have less abundance of caterpillars than non-Bt fields, resulting in lower abundance of generalist arthropod predators and enhancing more stink bug pressure.
2. Predator and herbivore abundance in field margins are positively associated with their abundance inside soybean crops.
3. Annual crops in the landscape are negatively associated with the abundance of arthropod predators in soybean fields, whereas perennial crops, natural grassland, and native and commercial forests have a positive association with predator abundance.
4. The response of generalist predators and herbivores to the landscape and local variables in soybean is affected by crop phenology and it could be evaluated differently with beating sheet and sweep net

2. Materials and methods

2.1. *Study area and sites*

The study was performed during the 2018 and 2019 summer seasons in the western coastal region of Uruguay (Fig. 1a). The area belongs to the West Sediment Basin (Brazeiro et al., 2012) with a humid subtropical climate, 1300 mm of annual rainfall, average median temperatures between 7.1 °C and 32 °C (extreme temperatures between 42 °C in mid-January and −4.5 °C in early July), and an altitude between 5 and 160 m (asl). The soils are deep, with very high natural fertility, traditionally dominated by a production system that rotates between agricultural crops and pastures for livestock grazing. In recent years, a continuous crop system has been established in the areas with higher soil potential, predominantly soybean crops and a significant expansion of forestation (*Eucalyptus* spp.). Natural grassland cover an important area, with a dense tapestry associated with arboreal park vegetation and a predominance of Fabaceae shrubs.

A total of 60 sites were sampled over a period of two soybean crop cycles ($n = 32$ in 2018 and $n = 28$ in 2019, Fig. 1b, Table A1). In each year, about half of the selected commercial soybean fields were RR and non-Bt (Roundup Ready RR1®, hereinafter "non-Bt"), while the other fields were RR and Bt cultivars (Intacta RR2 PRO®, event MON 87701 x MON 89788, hereinafter "Bt") (Fig. 1b). All the sampled fields

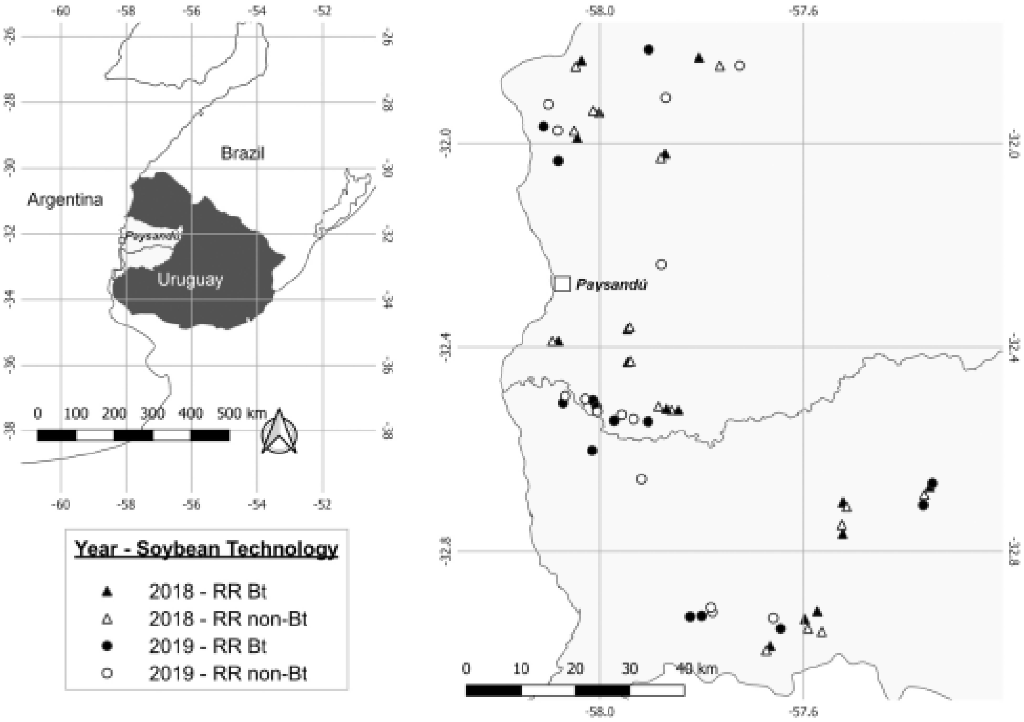

Fig. 1. a. Study region in the western coastal region of Uruguay; **b.** Sampled fields (2018 and 2019) discriminated by soybean technology.

corresponded to single soybean (sown in December) and none of them were irrigated. Fields sampled in 2018 were not repeated in 2019.

Sampled fields were located in a gradient ranging from 16.8% to 91.6% of the landscape sown with annual summer crops in a 1 km radius buffer (Fig. 1, Table A2).

Arthropod abundance was analyzed separately considering two crop phases to take into account crop phenological variations associated with the occurrence of pods (Fehr and Caviness, 1977): before beginning pod (included till R2, hereinafter "BEFORE R3") and after beginning pod (from R3 to harvest, hereinafter "AFTER R3").

2.1.1. *Insecticide management in Bt and non-Bt fields*

Caterpillars may damage soybean plants during the whole crop cycle, whereas stink bugs mainly cause damage during the crop's reproductive stage. Since the Cry1Ac protein effectively controls the predominant caterpillar species farmers usually do not apply insecticides during the vegetative stage in Bt fields. In non-Bt fields, farmers usually apply insecticide once or twice to control caterpillars. Despite the Bt technology used, two or three insecticide sprayings are needed to control stink bugs during the reproductive stage.

2.2. *Arthropod sampling*

2.2.1. *Soybean*

The arthropods (predators and herbivores) in Bt and non-Bt fields were sampled every three weeks during the whole crop cycle, from January to April each year (Table A1). A geo-referenced point was located in each field at 40 m from one margin (Woltz et al., 2012). Arthropod density in each field was estimated using a vertical beating sheet (Drees and Rice, 1985). The method consisted in beating soybean foliage against a 1 m long white vertical beating-sheet attached to a plastic trough. The vertical beating sheet is used to estimate the density of leaf-dwelling insects for pest management, as action thresholds in South America are frequently calculated in units of density. Four samples were taken at each geo-referenced point within a 15 × 15 m sampling plot. Arthropod abundance was estimated with a sweep net (⌀ 38 cm). A sweep net can sample many different crop rows and is capable of catching large quantities of above-ground insects per sample with minimal damage to plants (Kogan and Pitre, 1980). From the two opposite vertices of the sampling plot, two transects of 15 m were defined in the same direction of the crop rows. The arthropods were collected by sweeping 15 times in each transect. One sample was obtained for each transect and they were kept at −18 °C until laboratory sorting. Insects were identified at order, genus or species level depending on their state of conservation. For both sampling methods (sweep net and beating sheet), all the collected predators (immature and adults) were considered. Among herbivores, only caterpillars and stink bugs species (immature and adults) were identified. The number of insecticide sprays and the active ingredients used were also recorded.

2.2.2. *Margin*

The neighboring habitat immediately adjacent to the soybean field perimeter, constituted by strips of spontaneous herbaceous vegetation (a mixture of winter and summer grasses, legumes, broad leaf weeds and sedges) with a width range between 1 and 4 m, was determined as a margin. The closest margin to the geo-referenced field point was sampled (40 m). From that margin, two transects were defined in the same direction as the herbaceous vegetation. The arthropods were collected by net sweeping 15 times in each transect. Insect samples were kept at −18 °C until laboratory identification at order, genus or species level depending on their state of conservation. Spiders were considered as a whole and not identified by family, genus or species.

2.3. *Local field variables and landscape composition*

Local variables included soybean technology (Bt and non-Bt), perimeter to area of the soybean fields (calculated using QGIS software), abundance of potential predators (for the study of herbivore species) or potential prey (for the study of predators) (Tables A3 and A4, respectively), and abundance of each herbivore/predator species/group in field margin.

Landscape composition was quantified using Google Earth satellite images for 2018 and 2019, followed up by ground-truthing for each site (Midega et al., 2014). The landscape variables identified in the field inspection were grouped into eight categories: soybean, maize and sorghum (summer annual crops), natural grassland, native forest, commercial forest, prairie with legume and prairie without legume (Tables 1 and A2). The QGIS software program (QGIS Development Team, 2018) was used to delimit a 1 km radius around each site. Landscape diversity was characterized according to the Shannon index, where the different landscape elements were expressed as a function of the proportional abundance (Table A2).

Landscape composition was conducted once during the whole soybean crops, since there are no relevant changes in the vegetation cover during the summer period in the studied region.

Table 1

Local and landscape variables in a 1 km buffer area of the sampled soybean fields used in this study.

Categories	Variables	Description
Local variables	Soybean technology	Insect resistance technology of soybean (Bt and non-Bt)
	Herbivores in margins	Abundance of herbivores (Stink bugs and Noctuidae) in the field margin (see Tables A3 and A5)
	Predators in margins	Abundance of predator (Spiders, Neuroptera, Hemiptera, Coleoptera) in the field margin (seeTables A4 and A6)
	Preys in soybean	Abundance of the main prey by each predator group in the sampled soybean field (seeTables A3 and A5)
	Predators in soybean	Abundance of the main predators by each herbivore group in the sampled soybean field
	Perimeter/Area	Perimeter-to-area ratio of the sampled soybean field (m^{-1})
Landscape composition	Soybean	Proportion of soybean cover
	Maize	Proportion of maize cover
	Sorghum	Proportion of sorghum cover
	Prairie with legume	Proportion of prairie with legume cover (mainly alfalfa)
	Prairie without legume	Proportion of prairie without legume cover (mainly *Festuca* sp., *Lolium* sp.)
	Natural grassland	Proportion of natural herbaceous vegetation cover (winter grassland: *Briza* sp., *Bromus* sp., *Lolium* sp., *Piptochaetium* sp., *Poa* sp. and *Stipa* sp., summer grassland: *Andropogon* sp., *Axonopus* sp., *Bothriochloa* sp., *Bouteloua* sp., *Coelorhachis* sp., *Cynodon* sp., *Digitaria* sp., *Eleusine* sp., *Eragrostis* sp., *Paspalum* sp., *Schizachyrium* sp., *Setaria* sp. and *Sporobolus* sp.; legumes: *Trifolium polymorphum*, *Adesmia bicolor*, *Desmanthus depressus*, *Desmodium* sp.; broad leaf weeds and sedges)
	Native forest	Proportion of forest cover (mainly *Fabacea*: *Acacia cavens*, *Prosopis affinis*, *Prosopis nigra*, *Parkinsonia aculeata*; Anacardiaceae: *Schinus molle*; Pittosporaceae: *Pittosporum undulatum*)
	Commercial forest	Proportion of forest cover (mainly *Eucalyptus* spp.)

2.4. *Statistical analyses*

We used Spearman rank correlations (Dormann et al., 2013) to test the degree of correlation between landscape composition and local field variables (Table A7). When variables were very strongly correlated, one of them was excluded from the analysis, such as the Shannon index that was strongly correlated with soybean (Table A7, Campbell and Swinscow, 2009; Schmidt et al., 2019).

The effects of the landscape composition and local variables on predator and herbivore abundance (sweep net and beating sheet) on soybean were analyzed at two crop phases, before beginning pod (BEFORE R3) and after beginning pod (AFTER R3). To simplify data interpretation, we grouped the predators into spiders, Hemiptera, Coleoptera and Neuroptera and the herbivores in two groups, stink bugs and caterpillars. We used a linear mixed-effects model with the year as the random structure for each model using the "nlme" package (Pinheiro et al., 2021) for the R software (R Development Core Team, 2018). For each field and margin, the abundance of the samplings carried out BEFORE R3 and AFTER R3 were averaged for each year to obtain a mean number for each phase. The mean number of each arthropod group was log transformed [$\log10(x + 1)$] to achieve, as much as possible, a normal distribution of the model residual. We tested the spatial autocorrelation in the mean abundance of each arthropod group between fields (BEFORE R3 and AFTER R3) using Moran`s I statistic (Paradis, 2019) (Tables A8a and A8b). Some groups showed a spatial autocorrelation, so we ran the linear mixed-effects model with a Gaussian spatial correlation structure, corGaus for lme (Pinheiro et al., 2021). Landscape metrics for each model were standardized (mean centered and scaled) using the ""caret" package (Max et al., 2021). We applied a multi-model inference approach to obtain a robust parameter estimate using the "MuMIn" package (Bartoń, 2020). The dredge function of the models was used to describe the effects of independent variables on each dependent variable. Models were selected by comparing the Akaike information criterion corrected for small sample sizes (AICc) with the values of the full model. Model averaging was performed on the model set with $\Delta AiCc < 2$ (Burnham and Anderson, 2004). The model residuals were graphically inspected with Q-Q plots and histogram graphics to ensure there were no violations of normality and homoscedasticity assumptions (Zuur et al., 2010). We used the "effects" package (Fox et al., 2020) to represent the effects in partial residual plots.

3. Results

3.1. *Arthropods sampled in soybean*

Over the two years of the study, a total of 6863 arthropods were collected using a sweep net (52.8% of the total recorded) and a beating sheet (47.2% of the total recorded) in the 60 sampled soybean fields: 4734 in 2018 ($n = 32$ fields) and 2129 in 2019 ($n = 28$ fields). Predators represented 63.2% of all recorded specimens (67.1% sweep net and 59% beating sheet). Stink bugs and caterpillars, the herbivores considered for the analysis, accounted for 26.2% and 10.6%, respectively, of the total arthropods recorded. The abundance of the different species/groups of arthropods was similar between Bt and non-Bt soybean fields (Tables A5 and A6), except for caterpillars, which were more abundant in non-Bt fields (Table A6). The species recorded with both sampling techniques were the same except for the coccinellid predator *Hyperaspis festiva* Mulsant (Col. Coccinellidae), which was captured only with the sweep net (Table A5). Predators were prevalent in each sampled date taken BEFORE R3, while herbivores (stink bugs and caterpillars) were predominant AFTER R3.

Among the recorded predators, the order Araneae were the most abundant (59.8%) while the predominant identified taxa of insects belong to Hemiptera (*Orius* spp. [Anthocoridae], *Geocoris* spp. [Geocoridae], and *Nabis* spp. [Nabidae]), Neuroptera (*Chrysoperla externa* Hagen [Chrysopidae]), Coleoptera (mainly *Eriopis connexa* Germarar, but also *Scymnus loewii* Mulsant, *H. festiva*, [Coccinellidae] and *Lebia conncinia* Brulle [Carabidae]). The few collected specimens of Syrphidae (Diptera), and Mantodea were excluded from analysis.

Piezodorus guildinii Westwood (Hemiptera: Pentatomidae) was the most abundant stink bug recorded both years, accounting for 72% of total herbivorous considered in the study (Table A6). Other abundant stink bugs were *Dichelops furcatus* Fabricius (15%), *Edessa meditabunda* Fabricius (11.5%) and *Nezara viridula* Linnaeus (1%). Among caterpillars, the velvetbean caterpillar (*Anticarsia gemmatalis* Hübner) was the most abundant species, followed by *Rachiplusia nu* Guenée and *Chrysodeixis includens* Walker. *Spodoptera* species were the minority, mainly *Spodoptera cosmioides* Walker.

3.2. Arthropods sampled in soybean field margin

A total of 4491 arthropods were collected in field margins over the two years of the study, mainly predators (92.5%). Herbivores represented the remaining 7.5% (4.6% stink bugs and 2.9% caterpillars). Spiders were the predominant group between predators (81.9%, Table A5). In Bt and non-Bt soybean fields, spiders were predominant in field margins at the two phenological soybean phases (BEFORE R3 and AFTER R3) (Table A6).

The significant landscape variables are presented in Tables 2 and 3 for predators and herbivores, respectively, and the most parsimonious models for predators and herbivores are shown in Tables A9a, A9b and A10a, A10b, respectively. Only the most significant associations of local and landscape variables with sampled arthropods were represented graphically.

3.3. Local variables

3.3.1. Soybean technology

The abundance of caterpillars during the whole soybean cycle was determined by crop technology (Table 3) and it was lower in Bt soybean fields (Fig. 2). Neither predator nor stink bug abundance presented an association with soybean technology.

3.3.2. Arthropod abundance in field margins

The abundance of many groups of predators in soybean fields was positively associated with their abundance in the margins (Table 2; Fig. 3), including spiders (BEFORE R3 and AFTER R3), Coleoptera (BEFORE R3), and Neuroptera (AFTER R3). Herbivores abundance was not affected by its abundance in the margins.

3.3.3. Prey-predator abundance

The abundance of spiders, Coleoptera and Hemiptera predators in soybean was positively associated with the abundance of their prey in both sampled crop phases (Table 2). Stink bug sampled in soybean presented a positive association with their main predators AFTER R3 (Table 3).

3.3.4. Relation perimeter/area

Fields with higher perimeter/area ratio presented a negative relation between the abundance of Neuroptera AFTER R3 (Table 2).

3.4. Landscape variables

The association between landscape variables and the predators or herbivores was influenced by a temporal factor of the crop (BEFORE R3 vs. AFTER R3). Regarding the sampling technique, more relations were identified when using the sweep net, although the results in most cases coincided with those of the beating sheet sampling (Tables 2 and 3).

3.4.1. Annual crops: soybean, maize and sorghum

The proportion of soybean in the surrounding landscape was the landscape variable with more significant associations with predators, negatively determining the abundance of Neuroptera (BEFORE R3), spiders and Hemiptera (AFTER R3) in soybean (Table 2, Fig. 4).

The proportion of maize in the landscape was only positively with spiders AFTER R3.

The proportion of sorghum in the landscape showed a positive association with the abundance of Coleoptera but negatively associated with spiders AFTER R3 (Table 2).

3.4.2. Perennial crops: prairie with and without legume

The proportion of prairies with legumes presented a positive association with neuropterans (BEFORE R3) a negative association with spiders AFTER R3 (Table 2).

Neither predator nor herbivore abundance was significantly associated with the proportion of prairie without legume in the landscape (Tables A9a, A9b, A10a and A10b).

3.4.3. Natural grassland

The proportion of natural grassland in the landscape showed a positive association with the abundance of the most frequent predators in soybean: spiders, Coleoptera and Hemiptera (Fig. 5) (Table 2).

3.4.4. Native and commercial forest

The abundance of predators was not affected by the proportion of native forest (Table 3). However, we found a positive association with stink bugs during both sampling phases (Table 2).

The abundance of spiders in soybean was positively associated with commercial forestry plantations during the two sampled phenological periods (Table 2).

4. Discussion

As we hypothesized, Bt soybean field have effectively less amount of lepidopteran larvae, but this not determinate a decreased in predators abundance. The abundance of predators in soybean fields was highly associated with their abundance in the field margins, indicating the importance of spontaneous vegetation cover from margins for biological control. In opposite to our hypothesis, our results provide evidence about the differential effect of annual crop included in the landscape to the abundance of the different arthropod groups. We observed a negative effect of the proportion of soybean in the landscape on the abundance of the most frequent predators present in soybean fields, such as spiders. Contrary, non-annual crops and native grassland present the highest number of positive associations with predators. Sorghum in landscape present a positive effect on coleopterans but a negative effect on spiders of soybean field. Herbivores were less affected by landscape variables compared with predators.

Since different sampling methods allow us to identify different species of pests and natural enemies (Schmidt et al., 2008; Gill and O'Neal, 2015), we estimated the abundance of arthropods with two complementary methods: a vertical beating sheet and an entomological sweep net. The two methods allowed us to find many of the same significant associations between some groups/species and local/landscape variables, which is an indicator of the robustness of the data we obtained and the complementary nature of the two methods. We obtained more relations with the sweep net, probably because a larger sampling area was covered with this method, making it more efficient. Sweep net allowed sampling the spontaneous vegetation of margins while beating sheet is not adequate to collected arthropods from low height vegetation cover.

4.1. Local variables

4.1.1. Bt technology

In South America, *A. gemmatalis* and the semi-loopers *R. nu* and *C. includens* are considered major lepidopteran leaf-feeding pests in soybean crops (Bueno et al., 2021) and they were predominant in our study. Our data confirm that in Uruguay, the Cry1Ac expression in

Table 2

Significant variables (p values ≤ 0.05) in the best models ($\Delta AIC < 2$) relating predator abundance with landscape and local variables. Variables are standardized (mean-centered and scaled). Relative importance is the sum of Akaike's weight associated with the variables in the best models. BS = beating sheet; NET = sweep net.

			Before R3							After R3						
	Variable	Species/ Group	Estimate	Std. Error	Adjusted SE	z value	Pr (>|z|)	Rel. imp.	Samp. Method	Estimate	Std. Error	Adjusted SE	z value	Pr (>|z|)	Rel. imp.	Samp. Method
Local	Abundance in field margins	Spiders	0.22	0.09	0.09	2.33	0.020100	1	BS	0.26	0.06	0.06	4.19	0.000028	1	BS
			0.33	0.09	0.09	3.61	0.000312	1	NET	0.23	0.08	0.09	2.7	0.007000	1	NET
		Neuroptera								0.31	0.09	0.09	3.28	0.001029	1	NET
		Coleoptera	0.83	0.26	0.27	3.11	0.001880	1	BS							
			0.33	0.14	0.14	2.30	0.021500	1	NET							
	Prey abundance	Spiders								0.23	0.07	0.07	3.47	0.000522	1	BS
			0.2	0.09	0.1	2.08	0.037369	0.84	NET	0.18	0.08	0.08	2.19	0.028472	1	NET
		Coleoptera								0.26	0.07	0.07	3.59	0.000337	1	NET
		Hemiptera								0.25	0.10	0.10	2.62	0.008920	1	BS
			0.25	0.11	0.11	2.17	0.029800	0.91	NET	0.32	0.09	0.09	3.60	0.000317	1	NET
	Perimeter/Area	Neuroptera								-0.1	0.04	0.04	2.18	0.029292	1	NET
Land-scape	Soybean	Spiders								-0.26	0.06	0.06	4.08	0.000044	0.35	NET
		Neuroptera	-0.19	0.07	0.07	2.65	0.008130	0.08	NET							
		Hemiptera								-0.17	0.07	0.07	2.35	0.018590	0.71	BS
	Maize	Spiders								0.13	0.06	0.06	2.14	0.0325770	0.65	NET
	Sorghum	Spiders								-0.11	0.05	0.05	2.26	0.024141	0.93	BS
		Coleoptera	0.14	0.06	0.06	2.36	0.018300	1	NET							
	Prairie with legume	Neuroptera	0.16	0.07	0.07	2.37	0.017900	1	BS							
		Spiders								-0.16	0.06	0.06	2.41	0.015861	0.35	NET
	Natural grassland	Spiders								0.14	0.05	0.05	2.86	0.004276	1	BS
										0.21	0.06	0.06	3.50	0.000462	0.65	NET
		Coleoptera								0.11	0.04	0.04	2.59	0.009510	1	BS
		Hemiptera								0.14	0.07	0.07	1.99	0.046980	0.29	BS
	Commercial Forest	Spiders	0.14	0.07	0.07	2.01	0.044600	0.9	BS							
			0.16	0.07	0.07	2.46	0.013807	1	NET							

Table 3
Significant variables (p values ≤0.05) in the best models (ΔAIC<2) relating herbivore abundance with landscape and local variables. Variables are standardized (mean-centered and scaled). Relative importance is the sum of Akaike's weight associated with the variables in the best models. BS = beating sheet; NET = sweep net.

	Variable	Specie/ Group	Before R3							After R3						
			Est	Std. Error	Adjusted SE	z value	Pr (>|z|)	Rel. imp.	Samp. Method	Estimate	Std. Error	Adjusted SE	z value	Pr (>|z|)	Rel. imp.	Samp. Method
Local	Soybean technology	Noctuidae								0.78	0.15	0.15	5.21	2.00E-07	1	BS
	(Bt, non-Bt)		0.6	0.15	0.15	3.91	0.000093	1	NET	0.48	0.12	0.12	4.00	0.000062	1	NET
	Predator abundance	Stink bugs								0.51	0.14	0.14	3.57	0.000363	1	BS
Landscape	Native forest	Stink bugs	-0.13	0.04	0.04	3.06	0.002150	0.25	NET	0.16	0.07	0.07	2.29	0.022100	1	NET

soybean is effective in controlling these pests, as it was previously reported for *A. gemmatalis* and *C. includens* in this country (Blanco et al., 2016) and in Brazil (Bernardi et al., 2012; Horikoshi et al., 2021). Although a resistance to Cry1Ac by *R. nu* was recently observed in Brazil (Horikoshi et al., 2021), in our study the abundance of the semi-looper caterpillars *R. nu* and *C. includens* was practically insignificant during both periods in Bt fields. Another concern for Bt crops is the increased abundance in secondary pests (Martins-Salles et al., 2017). An increase in the negative impact of hemipteran species has been documented in certain Bt crop systems as a result of the decrease in the application of broad-spectrum insecticide (Lu et al., 2010). However, contrary to our hypothesis in our study we did not record an increase in stink bug pressure.

The environmental benefits of adopting Bt crops have been widely reported because of the reduction in the need for chemical applications (Romeis et al., 2006; Lu et al., 2012; Guo et al., 2016; Qaim, 2020). Conversely, the possible side effects on non-target organisms, such as the natural enemies, are a reason for concern, but most of the scientific literature shows no negative effects of them (Comas et al., 2014; Tian et al., 2014; Romeis et al., 2019). Nevertheless, most of the available literature refers to maize and cotton systems, while there is less information about possible side effects in Bt soybean crops. Regarding the effect on non-target arthropods, Yu et al. (2014) in a two-year study in China, and Marques et al. (2018) and Luz et al. (2021) in Brazil, taking into account similar predator groups to those considered in our study, determined no significant differences of predator abundance between Cry1Ac and Cry1F Bt soybean and its isoline. However, these studies were performed in small plots with few replications, which might not necessarily reflect what is happening in commercial field conditions. Our findings came from standard sized commercial fields in the soybean production system of Uruguay. Contrary to our hypothesis, these findings suggest that there is no negative effect on the predominant predators associated to the adoption of Bt technology at a commercial scale. This occurs despite the fact that Bt crops effectively host less available lepidopteran larvae, which are an important food source for the generalist predators (de Sene Pinto and de Freitas Bueno, 2019). This is likely associated with the occurrence of alternative food sources such as thrips and spider mites. In particular, this could have been important during the first sampling year when the low rainfall enhanced the development of this secondary pest (Table A11) in Bt and non-Bt fields.

4.1.2. Margins arthropod's abundance and relation perimeter/area

The number of associations found between the abundance of some predator groups (spiders, Neuroptera and Coleoptera) inside the soybean crops and their abundance in the field margins enables us to partially confirm our hypothesis, but we discard this association in the case of herbivorous insects. The ecological role of the margins has been reported for other regions and crop systems (Thies and Tscharntke, 1999; Mkenda et al., 2019; Clemente-Orta et al., 2020), but it has not been studied extensively in the Rio de la Plata region. The increase of areas under continuous agricultural systems in Uruguay has promoted the elimination of field margins due to the increased size of the actual fields. Therefore, it is of great interest to demonstrate the ecological function of the margins as a source of natural enemies. Generalist predators could benefit from field margins as overwintering sites, sources of alternative food or permanent refuge from adversities such as the application of insecticides (Landis et al., 2000; Denys and Tscharntke, 2002; Marshall and Moonen, 2002) or the field practices. Pest suppression was associated with the colonization of spiders from the margins towards the soybean crop, promoting re-colonization after pesticide applications, since they the margins act as shelter areas (Beltramo et al., 2006).

The variable perimeter/area appears to be important in the determination of biological control. It was reported that natural enemies benefit from small field areas with a high perimeter proportion because they can reach the crop field more easily (Haan et al., 2020). In our

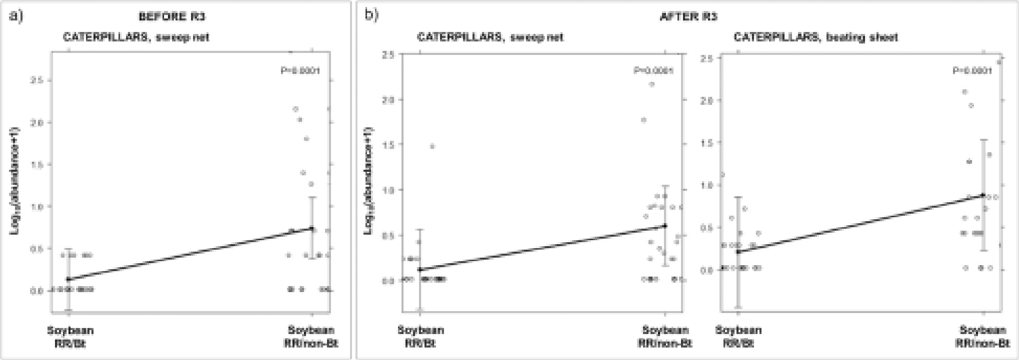

Fig. 2. Effects of the Bt technology (BEFORE and AFTER R3) on the number of Noctuidae caterpillars in soybean fields, sampled with vertical beating sheet and sweep net.

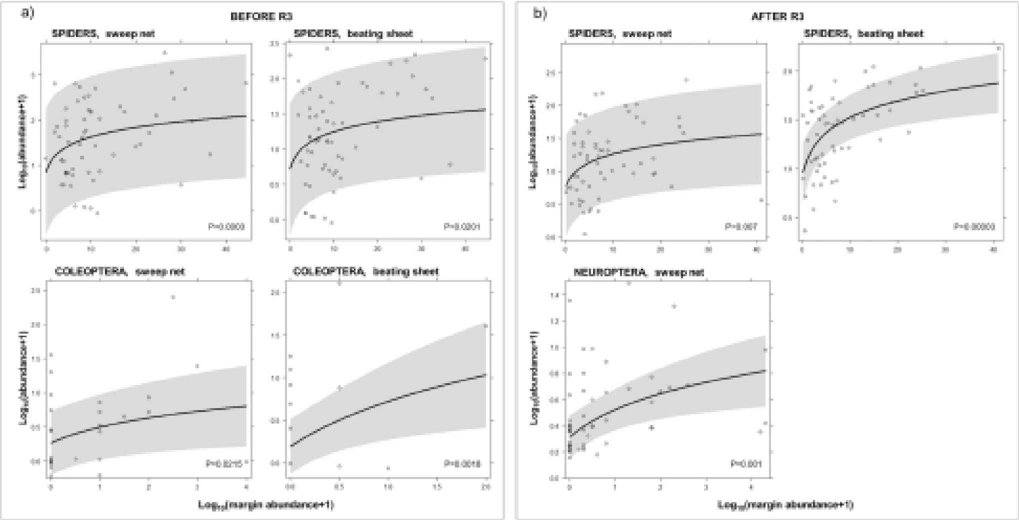

Fig. 3. Effects of the field margins abundance on the number of predators in soybean fields, sampled with vertical beating sheet and sweep net sampled BEFORE R3 (a) and AFTER R3 (b).

results, a negative association between the perimeter/area relation was detected for neuropterans AFTER R3, which could be associated with a greater migrating capacity from the crop when the conditions were not optimal, as it happens during the senescence. A positive association between the abundance of predators or pest suppression and perimeter/area was previously reported by other authors in different agroecosystems (Maisonhaute et al., 2017; Clemente-Orta et al., 2020). No associations between the abundance of herbivores in the margin and the number inside the soybean fields was detected confirming other studies (Macfadyen et al., 2015; González et al., 2016) and suggesting that natural enemies benefited from natural vegetation more than herbivores.

4.1.3. Prey-predator abundance relation

As it was expected, the abundance of spiders, Coleoptera and Hemiptera predators in soybean was favored by the abundance of their potential prey, which demonstrates the numerical responses of these two groups of arthropods, a feature reported by Pons et al. (2005) and Meseguer et al. (2021) for *Nabis provencalis*, *Orius* spp. and coccinellids. In a similar sense, the abundance of stink bug in the crops was positively affected by the abundance of their potential predators, in line with other studies (Madeira et al., 2021; Clemente-Orta et al., 2020). This type of relationship between pests and their natural enemies is a key consideration for chemical control, highlighting the importance of maintaining pest populations below the action thresholds without pretending to eradicate them completely.

4.2. Landscape

4.2.1. Annual crops

The increase of soybean in the landscape area occurs in a very significant portion of South America at the expense of removing non-crop areas and turning crop-grazing rotation systems into continuous agricultural systems. It is widely reported that the loss of vegetation diversity has a negative impact on natural enemies (Altieri and Nicholls, 2004; Venette, 2015; Balmford et al., 2018), but the impact of soybean

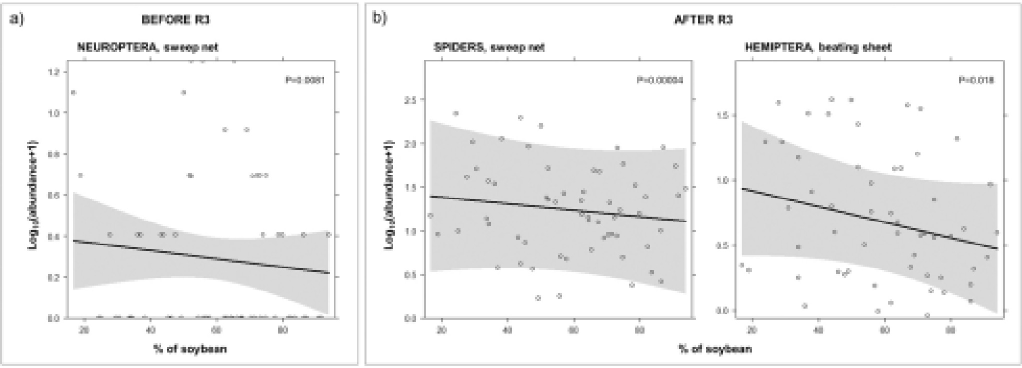

Fig. 4. Effects of the proportion of soybean (BEFORE and AFTER R3) in the landscape on the abundances of predators in this crop, sampled with sweep net and beating sheet.

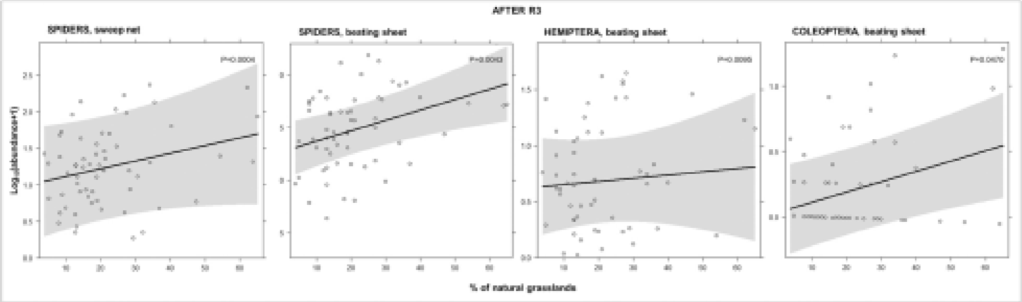

Fig. 5. Effects of the proportion of natural grassland (AFTER R3) in the landscape on the abundances of predators in natural grassland, sampled with sweep net and beating sheet.

adoption was not yet reported for Uruguayan agroecosystems from a landscape approach. Our results demonstrated that, just as we hypothesized, an increase in the proportion of soybean cover in a 1 km radius negatively affects the abundance of important groups of predators in soybean: spiders, Neuroptera and Hemiptera. Other studies previously reported that biological control in soybean fields is negatively affected in landscapes dominated by maize and soybean, compared with landscapes with an abundance of non-crop habitats (Gardiner et al., 2009). In Canada, natural enemies of *Aphis glycines* Matsamura (Hemiptera: Aphididae) in soybean were negatively influenced by the proportion of this crop cover in the landscape (Maisonhaute et al., 2017). Contrary to what we expected, a landscape dominated by soybean did not determine an increase in pest abundance. An association between higher proportions of annual crop acreage and pest pressures was reported in other regions (Andow, 1983; Tscharntke et al., 2005), while in Uruguay it was associated with a higher density of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in sorghum (Emery et al., 2021).

The abundance of spiders in soybean was also negatively affected by the proportion of sorghum in the landscape AFTER R3, but the cover of this summer crop was positively associated with coleopteran predators BEFORE R3. Sorghum in Uruguay is usually sown earlier than soybean. During its initial stages, it commonly hosts aphid and coccinellid populations and it is not frequently sprayed with insecticide. Therefore it is likely that coccinellids emigrate to soybean when the environmental conditions are not favorable for aphid populations in sorghum, as it was reported between sorghum and cotton (Prasifka et al., 1999; Tillman and Cottrell, 2012). A positive association between coccinellids and annual crops cover was reported by Emery et al. (2021). The number of spiders AFTER R3 was positively associated with maize cover, which could be explained because almost the entire area of maize sown in Uruguay has combinations of Bt events, so insecticide sprayings are not common (Blanco et al., 2016). This scenario could provide refuge conditions for spiders, which are quite sensitive to changes in agricultural systems (Sunderland and Samu, 2000).

4.2.2. *Perennial crops*

Prairies can be considered a habitat with an intermediate level of disturbance, between annual crops and areas such as natural grassland or native forest (Török et al., 2011), as they are subject to minimal chemical spraying, have longer production periods, and usually provide alternative food sources for predators. This could explain the positive associations obtained between prairie with alfalfa and neuropteran populations on soybean in the earliest stages of the crop. The role of alfalfa as a donor of heteropteran predators to annual crops has been previously reported (Madeira et al., 2014 and 2019). However, a negative association was detected between this vegetation cover and the number of spiders AFTER R3, this could be due to the scarcity of prey at this growth stages, trigging movement of spiders to alfalfa and acting as receptor habitats as observed for the maize predators (Madeira, and et al., 2014, 2019).

4.2.3. *Natural grassland*

In South America, a large area of natural grassland has been rapidly converted to croplands, although it is still the predominant land cover type in the Pampas (Baldi et al., 2006). Perennial plant species are predominant in these grassland, where disturbance is infrequent and the seasonal patterns of plant phenology differ from crop fields, determining a high abundance of predatory arthropods (Haan and Landis, 2019). In our study, we observed a positive association between spiders, coleopterans and hemipterans with the natural grassland, in line with Gardiner at al. (2009), who found the same association with a group of Coccinellidae. This result could indicate a spillover from these non-crop areas to the crop as previously reported for other predator species (Tscharntke et al., 2005; Blitzer et al., 2012).

4.2.4. Native and commercial forest

We observed a strong association between the abundance of spiders in soybean and in commercial forest (mainly *Eucalyptus*) BEFORE R3. This could indicate that these non-crop areas operate as a source of these predators, determining the early colonization of the crop. The movement of predators from woodlands to soybean crops was reported by Samaranayake and Costamagna (2019) and González et al. (2016), who determined that coleopteran predators move from native forests to soybean crops in greater numbers than vice versa. The movement of predators to soybean could decrease with senescence, which may explain the lack of associations AFTER R3. In contrast, herbivores were less affected by commercial forests. We detected a positive associations between the proportion of native forest in the landscape and the abundance of the stink bugs in soybean fields during the two periods evaluated. Adult stink bugs are in reproductive diapause in autumn and winter (Zerbino et al., 2016). Prospections made in Uruguay indicated that the main overwintering host plant for *P. guildinii* was *Pittosporum undulatum* Vent (Zerbino et al., 2020). The role of native forests in the population dynamics of the most frequent species of stink bugs could be associated with the niches providing the conditions for diapause. In the Chaco Serrano region of Argentina, González et al. (2017) also found a positive association between the abundance of herbivores during soybean flowering and the native forest cover in the landscape. In Uruguay, no insecticides are applied in commercial or native forests, so these areas would act as arthropod refuges. We could confirm this for spiders and stink bugs, suggesting that these non-crop areas should be considered for soybean pest management.

4.3. Soybean phenology

As we hypothesized, the response of generalist predators and herbivores to the landscape and the local variables was affected by temporal effects in soybean, as previously observed by González et al. (2016, 2017) for this crop and by Clemente-Orta et al. (2020) and Madeira et al. (2021) for maize and alfalfa. Although we recorded a greater number of predators in the period BEFORE R3, we found a higher association between local and landscape variables and arthropod abundance in soybean fields in the period AFTER R3. The decrease in the number of predators during the more advanced phenological stage of soybean could be associated with the use of broad-spectrum insecticides needed to control stink bugs (Abbate et al., 2022). The higher number of associations recorded during the most advanced period of crop development could be explained by the fact that in Uruguay most of the vegetation cover in the landscape is senescent, while soybean is still green and would serve as a refuge area for predators migrating from other habitats (Anggraini et al., 2021).

Stink bugs were more abundant in the period AFTER R3 and were associated with the emergence of soybean pods, which is their preferred food (Panizzi et al., 2000), but the number of associations with the landscape variables did not vary between phenological phases. An increase in herbivore abundance during the reproductive stage of soybean was already reported by González et al. (2016), who also reported a decrease in the abundance of predators towards the senescence of the crop. The similar number of associations between stink bugs and landscape variables in both sampled periods could be explained by a high dispersal ability (Sosa-Gómez, 2004). In our study, some effects may probably be misperceived, since the period AFTER R3 included a wide range of crop developmental changes (from pod emergence to soybean senescence) that probably affected arthropod species differently. Crop phenology determines the interchange of arthropod between annual crops and adjacent habitats (Rand et al., 2006).

5. Conclusions

This is the first study that determines the effects of changes in the landscape composition (the increase in annual crops) and local changes (the adoption of Bt technology) on the most frequent predators and pests of soybean in Uruguay. Results suggest that the relationships between local and landscape components and the quantity of predators and pests would depend on the following: i) the characteristics of each species or groups; ii) the type of vegetation cover, and iii) temporary variations associated to soybean phenology and environmental conditions. To detect landscape and local effects on soybean arthropods, it is recommendable to use both sampling methods because they are complementary and allow to increase the number of relationships identified.

Our more relevant conclusions were the following:

1. Local and/or landscape variables determined the abundance of the main frequent predators and pests of soybean in Uruguay.
2. The number of arthropod predators in soybean did not decrease with the adoption of Bt soybean, while Bt fields were associated with less caterpillars' abundance
3. Soybean field margins are an important source of arthropod predators for soybean fields, without the risk of also being a refuge for pest species.
4. Crops maize, natural grassland and commercial forest have a positive effect on the abundance of the main arthropod predators.
5. The increase of the soybean area in the landscape negatively affects many predator groups.

These findings provide information to better understand the impact of landscape homogenization and the adoption of Bt soybean technology on the most frequent predatory arthropods and insect pests of this crop, and our results can contribute to improving the sustainability of soybean pest management.

Ethical approval

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

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research is part of a project sponsored by Agencia Nacional de Investigación e Innovación ANII-Uruguay: Fondo María Viñas (FMV-2–2016-1–126142). SA obtained Postgraduate student grants by Agencia Nacional de Investigación e Innovación ANII-Uruguay (POS_NAC_2017_1_141080) and Comisión Académica de Posgrado CAP-Universidad de la República. Special thanks to all the cooperating farmers and Agricultural Engineers who provides the fields and all the information. We also thank field assistants and the many farmers and Agronomist who collaborated in this study.

Author contributions

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

Informed consent

The five authors of this manuscript agreed to submit the paper for publication in the journal Agriculture, Ecosystem and Environment, and report that this paper has not been published or accepted for publication in another journal, and it is not under consideration for another journal.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2022.108027.

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3. REFUGE AREAS FAVOR THE PRESENCE OF PREDATORS AND HERBIVORES IN BT SOYBEAN: A LANDSCAPE PERSPECTIVE

Abbate S, Madeira F, Bentancur O, Altier N, Pons X, Publicado online 17/11/2022, Journal of Pest Science. https://doi.org/10.1007/s10340-022-01580-7

Refuge Areas Favor the Presence of Predators and Herbivores in Bt Soybean: A Landscape Perspective

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Key words: insect-resistant GM crops, landscape composition, caterpillar pest, predators, *Glycine max*, insect-resistant management

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.

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 two 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 (1km 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 (IPM) in agroecosystems.

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 of 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; Tabashnik et al. 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 towards 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. 2010; 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 modelling 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; Samaranayake and Costamagna 2018; González et al. 2020). 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; Guedes et al. 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 does 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 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 x 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 to 765 meters 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, 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).

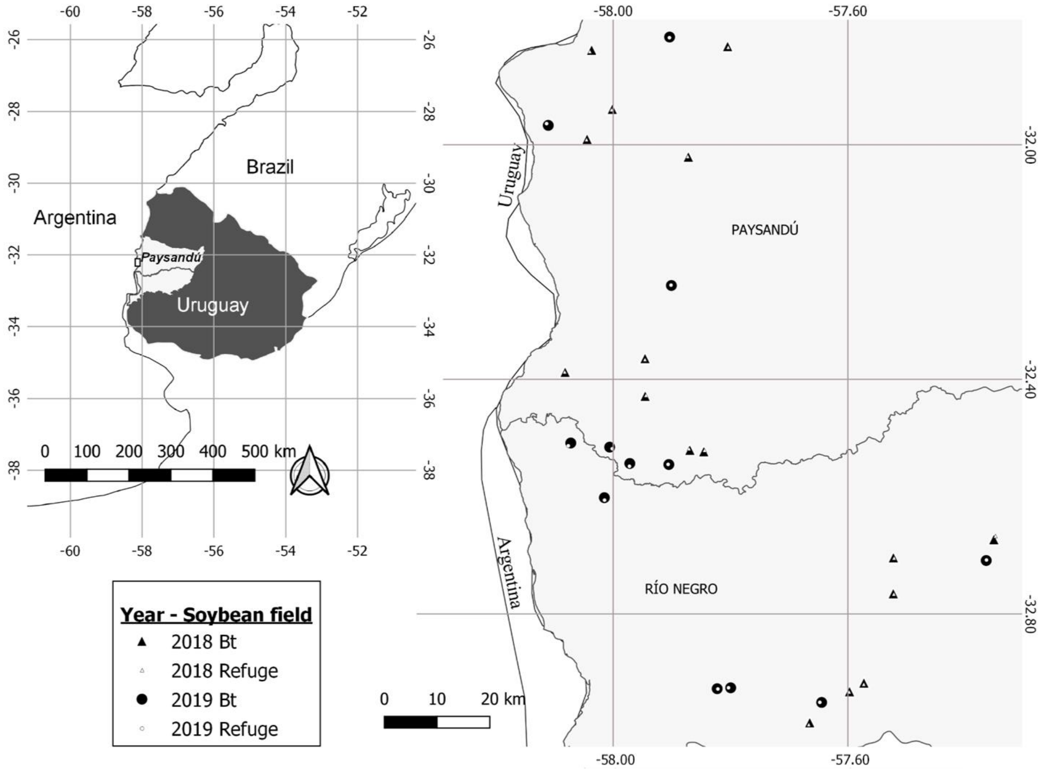

Fig. 1 Study region in the western coastal region of Uruguay **(a)**; Bt soybean sampled fields and non-Bt soybean refuge areas (2018 and 2019) **(b)**

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 three 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 (⌀ 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 two years of the study: 1498 in Bt soybean fields and 2123 in non-Bt 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).

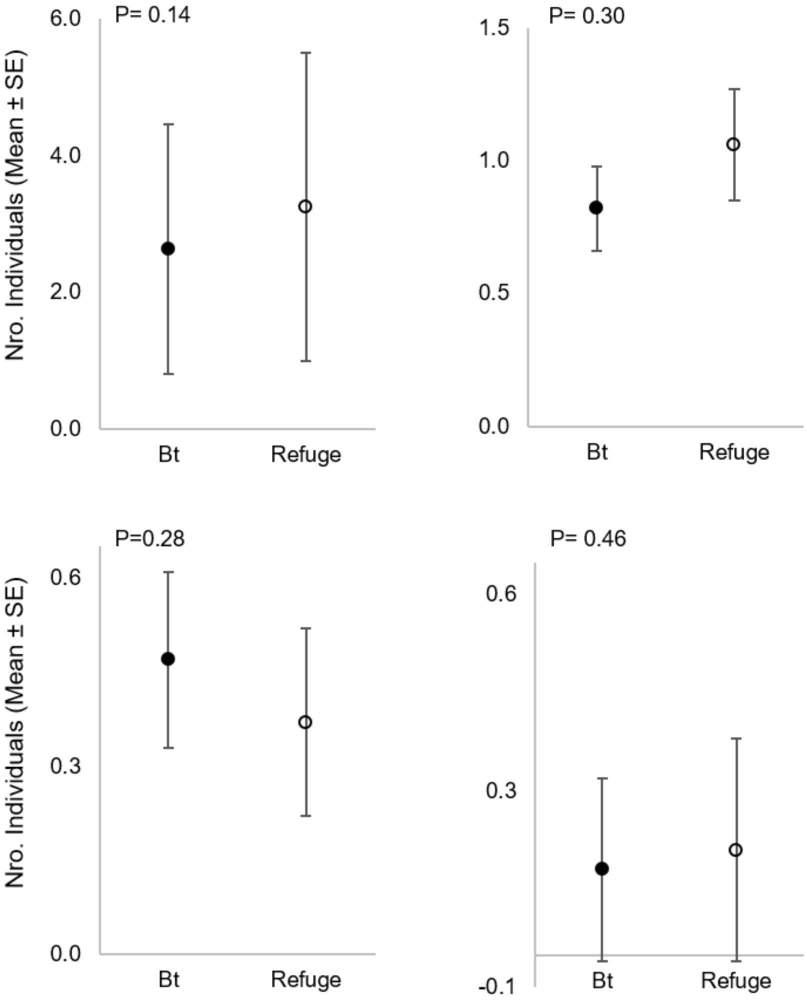

Fig. 2 Abundance of predatory arthropods: a) Araneae, b) Heteroptera, c) Chrysopidae, d) Coccinellidae, sampled in Bt soybean fields (solid circles) and non-Bt soybean refuges (empty circles). Values (mean ± standard error per 30 sweeps per sampled date) predicted by GLMMs. Significant differences between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

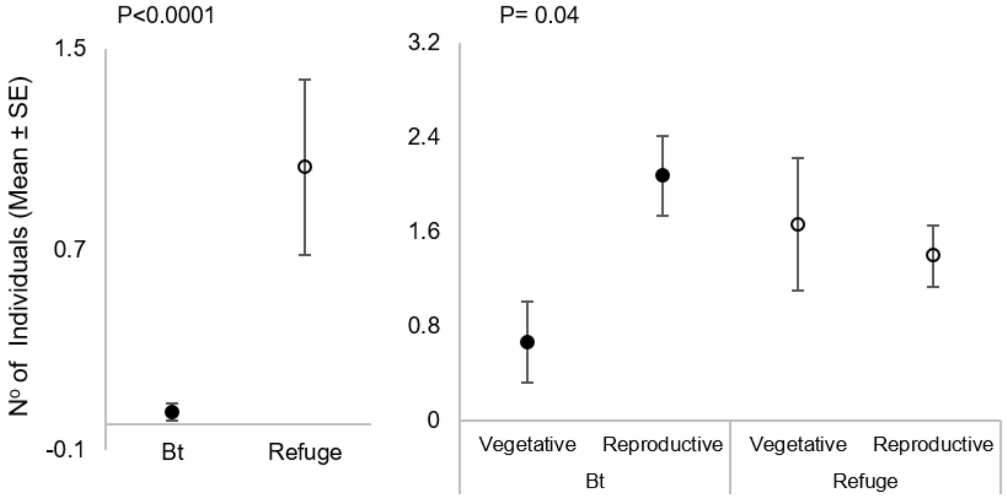

Fig. 3 Abundance of herbivore arthropods: a) caterpillars, b) stink bugs, sampled in Bt soybean fields (solid circles) and non-Bt soybean refuges (empty circles). Values (mean number of 30 times of sweep net per sampled date ± standard error) predicted by GLMMs. Significant differences between the technology's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

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 and b, Table S4), but it varied between the phenological stages within stink bugs, being higher during the reproductive period of the crop (Tables S4).

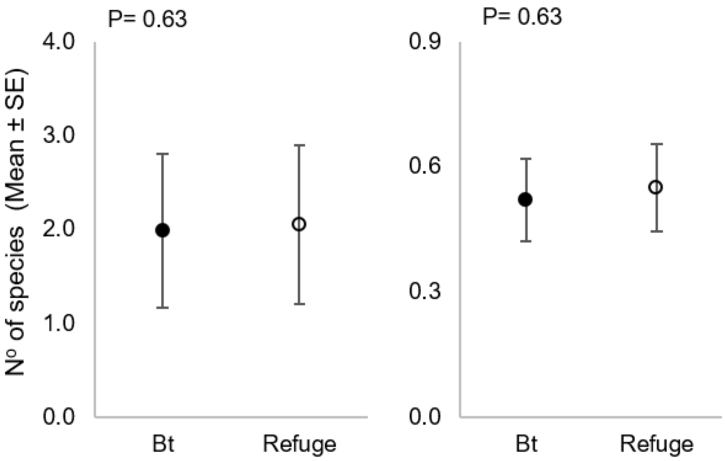

Fig. 4 Richness of predators (a) and stink bugs (b), sampled in Bt soybean fields (solid circles) and non-Bt soybean refuges (empty circles). Values (mean number of 30 times of sweep net per sampled date ± standard error) predicted by GLMMs. Significant differences between the technology's main effect,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

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

The increase of 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 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).

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)

		Abundance of Predators in Bt soybean				
Group	**Independent variable**	$\hat{\beta}$	$S_{\hat{\beta}}$	**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

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).

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)

		Abundance of Herbivores in Bt soybean				
Group	**Independent variable**	$\hat{\beta}$	$s_{\hat{\beta}}$	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

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).

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)

		Richness in Bt soybean				
Group	**Independent variable**	$\hat{\beta}$	$S_{\hat{\beta}}$	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

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 meters. 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 (Lin et al. 2014). Biological control, in conjunction with Bt crops, effectively suppresses the caterpillar pest population and decreases the development speed of resistant populations (Lin 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 of the abundance and diversity of natural enemies and with the decrease of 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 id 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 meters, 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.

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Acknowledgments: We give special thanks to MSc. H. Silva for his contributions, to all the field assistants and to all the cooperating farmers and Agricultural Engineers who provided the fields and all the information. We are especially grateful to field assistants. We also want to express our appreciation to Dr. W.D. Hutchison (University of Minnesota) and another anonymous reviewer for their valuable comments and suggestions for improving an earlier version of the manuscript.

Funding: This research is part of a project sponsored by Agencia Nacional de Investigación e Innovación (ANII, Uruguay): Fondo María Viñas (FMV-2-2016-1-126142). SA obtained Postgraduate student grants by ANII (POS_NAC_2011_1_3559) and Comisión Académica de Posgrado (CAP)-Universidad de la República (Uruguay). FM thanks national funding by the Foundation for Science and Technology (FCT, Portugal), through the institutional scientific employment program contract. The authors are grateful to the Foundation for Science and Technology for its financial support through national funds FCT/MCTES (PIDDAC), to CIMO (UIDB/00690/2020 and UIDP/00690/2020), and to SusTEC (LA/P/0007/2020).

Author Contribution Statement: 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.

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.

Availability of data and materials: Not applicable.

4. BT SOYBEAN EXPRESSING CRY1AC DOES NOT AFFECT THE DEVELOPMENT, REPRODUCTION OR FEEDING BEHAVIOR OF RED-BANDED STINK BUG *PIEZODORUS GUILDINII* (HEMIPTERA: PENTATOMIDAE)

Abbate S, Xavier Pons X, Altier N, Lucini T, Bonansea S, Bentancur O, Armand Pilón A, Silva H, De Souza G, Irigoyen L, Panizzi A. Será enviado a la Revista Neotropical Entomology.

Bt soybean expressing Cry1Ac does not affect the development, reproduction or feeding behavior of red-banded stink bug *Piezodorus guildinii* (Hemiptera: Pentatomidae)

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Abstract

Bt soybean crop is increasingly used worldwide. Although the presence of the Cry1Ac protein expressed in Bt soybean efficiently controls several lepidopteran pests, the effect on non-target arthropods is of concern. *Piezodorus guildinii* (Westwood) is a major pest for soybean throughout the Americas. It is known that Bt crops are not effective for its control, while sub-lethal effects are unknown. In this study, we determined through an ELISA detection test if *P. guildinii* is able to ingest Cry1Ac from Bt soybean and the possible effect on its development, reproduction, survival and feeding behavior, by comparing two near-isogenic soybean varieties: DM5958iPRO (Bt) and DM59i (non-Bt). Biological traits were evaluated under controlled conditions from third instar nymphs feeding with pods. The adults obtained were placed in pairs (31 per treatment) and their reproductive performance was evaluated. Survival was recorded during the whole cycle. Feed behavior was recorded using an AC-DC electropenetrograph (EPG) device. Although *P. guildinii* is able to ingest the protein, nymphal period and accumulated survival percentage did not differ between both treatments. Feeding with Bt pods did not affect the number of postures nor the number of eggs per female. Egg viability was also not affected. Different feeding behaviors were only detected between the pathway phase (stylet penetration deep into plant tissue), which could be associated with morphological differences between varieties. Food ingestion on leaves, stems and pods, salivation on pods and non-probing phases did not differ between treatments.

Key words *Glycine max*, Bt genetically modified crops, non-target pests, electrical penetration graph, integrated pest management, EPG.

Introduction

Soybean (*Glycine max* [L.] Merr.) is one of the five dominant crops in global agriculture and has become one of the most important commodities in global trade (Sun et al. 2018). This oleaginous crop provides the largest worldwide protein source for animal feed and the second largest source of plant-based oil consumption (United Soybean Board 2022). More than half of the world's soybean production is currently located in South America (FAOSTAT 2019), where one of the major constraints are yield losses and control costs for insect pests. In this region and for this crop, leaf-feeding caterpillars (Lepidoptera: Noctuoidea) and stink bugs (Hemiptera: Pentatomidae) are considered primary pests that cause economic yield losses (Bortolotto 2015; Bueno et al. 2021). Most caterpillars consume leaves throughout the whole crop cycle, whereas both stink bugs and a few species of caterpillars feed on pods and seeds (Panizzi et al. 2000; Bortolotto et al. 2015).

To prevent caterpillar damage, Bt soybean varieties (Intacta RR2 PRO®) has been commercialized in South America since 2012 and reached 34.4 million ha in 2020/2021 (Bayer 2022). Bt soybean contains the stacked event MON 87701 × MON 89788 that expressed the Cry1Ac insecticide Bt protein and the 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) protein of *Agrobacterium* sp. (Justiniano et al. 2014). On the other hand, the control of stink bugs is based exclusively on chemical-based strategies with broad spectrum insecticide products (Baur et al. 2010; Temple 2011). Among the most frequent pentatomid species, the red-banded stink bug, *Piezodorus guildinii* (Westwood), is the most harmful to soybean seeds in South America (Corrêa-Ferreira and Azevedo 2002; Zerbino et al. 2016). Even though pentatomids are not a target pest of Bt soybean, the widespread adoption of this technology could influence directly through unknown sub-lethal effects or indirectly by affecting the dynamics of their populations in the landscape (Panizzi et al. 2022).

Although Cry proteins are directly toxic only to a narrow spectrum of lepidopteran and coleopteran pests that feed by chewing the plants (Whalon and Wingerd 2003; Chougule et al. 2013), little is known about their sub-lethal effects on non-target organisms, such as changes in demographical parameters or feeding behaviors. Most of the efforts that address the potential side effects of Bt crops on non-target organisms have focused on beneficial arthropods, determining no harmful effect on them (Lu et al. 2012; Tian et al. 2014; Romeis et al. 2019). Although negative impacts have been most evident on phytophagous arthropods rather than on natural enemies and pollinators (Han et al. 2016), they have been less studied. It has been reported that the high adoption of Bt cotton reduced the use of broad-spectrum insecticides, contributing to the emergence of stink bugs and true bugs as major pests (Greene and Turnipseed 1996; Wu et al. 2002). The mass use of Bt maize in Brazil determined the increase of stink bug populations, causing the need for insecticide application for their control (Panizzi et al. 2022). Likewise, the reduction of caterpillar damage in Bt soybean has decreased insecticide spraying during the vegetative stage of the crop (Abbate et al. 2022a), probably determining the anticipation of pentatomid outbreaks. Contrarily, in conjunction with the adoption of Bt soybean in South America, *P. guildinii* has decreased its populations in field crops, suggesting that changes occurring in the agroecosystem landscape are greatly affecting this species (Panizzi et al. 2022). The changes in the dynamics of a major pest of soybean such as stink bugs could determine variations in the management and use of the action thresholds. Therefore, research on the interaction of non-target pests with Bt technology is of great theoretical and practical importance.

The toxicity of Cry proteins depends on a complex process that requires multiple steps. After the ingestion of the crystal proteins contained in the plant tissue, the proteins must be solubilized in the insect's midgut and activated by the proteolytic processing of the protoxin. For this, the protoxin must present a high affinity for binding with the midgut receptor, followed by the irreversible insertion of the toxin into the membrane (Jenkins et al. 2000). Hemipteran pests are not particularly susceptible to Cry proteins (Porcar 2009; Li et al. 2011; Chougule et al. 2012, 2013), which is probably associated with the lack of functional receptors for them (Chougule et al. 2012). Although in most cases toxicity is correlated with receptor binding, some reports have also evidenced binding in non-susceptible or resistant insects (Brandt et al. 2004). The potential effects of Bt on the feeding behavior of Hemipteran species have been scarcely studied because their sucking mouthparts prevent the observation of the feeding process (Schünemann et al. 2014). The electrical penetration graph technique (EPG) allows studying this behavior. In the EPG, piercing-sucking insect and plant are part of a simple electrical circuit where a low current flows through (Tjallingii 1978; Walker 2000; Lucini and Panizzi 2017a). The feeding behavior of pentatomids has been characterized using the EPG system by the identification of computer waveforms associated to probing and non-probing activities (Lucini and Panizzi 2016; Lucini et al. 2016; Lucini and Panizzi 2017b). These advances in the understanding of the feeding behavior of pentatomids will allow the adoption of EPG techniques to study the potential Bt side effects on them.

If an insect is able to ingest a Cry protein and the toxin binds to its midgut epithelium receptors, the toxin could affect its physiology. The expression of Cry1Ac proteins in Bt soybean pods could produce changes in non-target herbivore insects. *Piezodorus guildini* is one of the most harmful pests of soybeans in South America and, although Bt technology has been widely implemented to control caterpillars, there is no information about the potential direct or indirect effects on this important pest, which could generate changes in its dynamics in agricultural landscapes.

The aim of the study was to determine if *P. guildinii* is able to ingest the Cry1Ac protein expressing in Bt soybean pods and its effects on the insect's performance. We hypothesize that *P. guildinii* is able to ingest Cry1Ac from Bt soybean pods without affecting its development, reproduction performance, mortality and feeding behavior. To do this, we tried to detect the Cry1Ac protein in the insect and its feces, and assessed the potential side effects on the survival, development, reproduction and feeding behavior of *P. guildinii.*

Material and methods

Plants

Two soybean varieties were evaluated: non-Bt soybean DM59i (RR) and DM5958iPRO (RR/Bt INTACTA^{TM}, MON 87701 × MON 89788, 40-3-2). Seeds were sown into plastic pots (3 liters) filled with sterilized soil and kept at Dr. Mario A. Cassinoni Experimental

Station, Paysandú, Uruguay (EEMAC, 58°03’W, 32°55’S), under mesh roofs for shadowing. Both varieties received identical agronomic management, guaranteeing their requirements of soil humidity and nutrition and keeping them free of pests. Soybean pods were harvested manually with scissors when plants reached the R6 phenological stage (Fehr and Caviness 1977) and transported to the laboratory to feed the stink bugs. Before offering them to insects, pods were disinfected by immersing them in a 2% sodium hypochlorite solution for 1 minute, after which they were washed with abundant distilled water then placed to dry in the open air and finally stored in a refrigerator (4 ºC) until their use.

Insects

A colony of *P. guildini* was initiated from adults collected from alfalfa fields at the EEMAC, taken to the laboratory and reared in plastic boxes (20 x 25 cm) lined with filter paper. Insects were maintained under controlled conditions (26±0.5 ºC, 75±5% RH, 16:8 L:D) and fed with green pods of *Phaseolus vulgaris* (L.) and pre-hydrated grains of non-Bt soybean and water. The postures of each stink bug female were identified, and hatching nymphs were kept together with water until the third instar, when they were destined for Cry detections and biological evaluations.

Cry1Ac detection assessments

Third instar nymphs from this colony were used, since the first two instars do not feed and present a gregarious behavior, which reduces their individual mortality. Third instar nymphs were placed individually into plastic rearing containers (7 x 3 cm), lined with filter paper, and assigned to each of the two treatments, non-Bt soybean and Bt soybean, according to a complete randomized design. They were maintained under controlled conditions (26±0.5 ºC, 75±5% RH, 16:8 L:D). Each insect was provided with two soybean pods and a plastic vial containing water that was topped with a cotton plug. Soybean pods and water were replaced every 5 days, or earlier if considered necessary. Three-day-old adults were used to determine if stink bugs were able to ingest the Cry1Ac protein expressed in Bt soybean pods.

The ELISA detection test (Quickstix, EnviroLogix®, Portland, ME) was used to identify the presence of the transgenic protein in the digestive tract, body, feces and eggs of 60 stink bug adults. The same ELISA test was used to confirm the presence of Cry1Ac in Bt variety pods and its absence in non-Bt ones. After feeding during 10 days with non-Bt (RR) or Bt (RR/BT) soybean, stink bugs were killed by placing them in a freezer (-17 ºC) for 10 minutes. Insect dissection was performed under a 40X binocular loupe (Olympus SZ40), placing each individual on a black wax base of a Petri dish and using entomological pins, straight and curved Castroviejo scissors, following Panizzi's protocol (personal communication, Figure A1), and following the anatomical diagram of pentatomids of Amutkan et al. (2015). Insects were placed with the abdomen downwards, and the head and legs were removed first. Incisions were made at each vertex contiguous to the prothorax and the mesoscutellum was removed and discarded. Distilled water was used to facilitate handling. A transversal cut was made in the last segment of the abdomen to remove the external genitalia. Subsequently, a cut was made following the lateral connexivum from the first to the last abdominal segments to remove the abdomen sclerites. Finally, the digestive tube was removed and placed separately from the rest of the body. A completely randomized design was used. Cry1Ac detection in the digestive tract was tested in six replicates constituted by the digestive tubes of ten adults per replication. A specific test of the rest of the body was performed in five replicates, with the tissues of the body (without digestive tube, head, or extremities) of five adults for each repetition.

The presence of Cry1Ac in the feces of *P. guildinii* was assessed using the fecal residues contained in filter paper used during the 10 days of the feeding treatment. A portion of 0.15 grams of filter paper on which traces of feces were observed constituted each of the five replicates conducted. The same amount of filter paper without being exposed to insects was included as a control to ensure that the paper used did not contain traces of the protein. The possible transfer of Cry1Ac to the *P. guildinii* eggs was evaluated by using 15 eggs laid from a same female of the feeding treatment on each of the five replicates conducted.

The digestive tubes, bodies, feces and eggs were placed separately into sterilized Eppendorfs with an extraction solution, and were macerated and homogenized with a vortex for 2 minutes. The test strips were immersed in the solution for 30 minutes to determine the results by counting the visible lines (Figure A2). The test result was considered positive if the protein was detected and two bands were colored, which occurs if the sample contains a value greater than or equal to a 0.3% concentration of Cry1Ac (EnviroLogix 2013).

Statistical analysis

The results were analyzed using the confidence interval (95%) for the true proportion by the Wilson-Score method (Wilson 1927). The experimental unit corresponded to the sample contained in the Eppendorf with the different tissues evaluated (10 digestive tubes, 5 bodies, 0.15 grams of filter paper which traces of feces, 15 eggs) and the number of successes in the total number of evaluated samples was determined. This method is considered appropriate to study binary results with a small sample, in this case, the presence or absence of Cry1Ac (values 1 and 0, respectively).

Biological, reproductive and survival assessments

Piezodorus guildinii third instar nymphs were assigned to treatments in a completely randomized experimental design and 16 replicates (determined by mothers), with a total of 130 nymphs evaluated (65 in each treatment). Half the eggs from each female stink bug colony were assigned to one treatment (non-Bt) and the other half to the other (Bt), to prevent the mother effect from interfering with the treatment effect. Individual rearing conditions were the same as described for the Cry1Ac detection assessments. From third instar to the adult stage, survival and duration of each nymphal instar were registered daily. After reaching the last ecdysis, each adult stink bug was sexed and placed in pairs (31 pairs per treatment) with pods and water, maintaining their assigned treatments to evaluate adult longevity and reproductive performance (total number of eggs and egg clusters laid/female and egg viability). The number of egg clusters and the total number of eggs laid were recorded. To assess egg viability, the egg clusters laid by each pair of stink bugs were transferred into plastic rearing containers, kept in the same environmental conditions, and the number of nymphs that hatched from the eggs was counted.

Statistical analysis

We used a generalized linear model (GLIMMIX procedure) of the statistical package SAS On Demand for Academics version 9.04 (SAS Institute Inc., Cary, NC, USA, 2018). The duration of each nymph instar and life span (from nymph hatching to adult death) was analyzed assuming a gamma distribution with *natural logarithm* as link function. Models included the treatment (Bt/non-Bt) and mother as fixed effects. The stink bug survival probability of each instar and treatment were analyzed using the confidence interval (95%) for the true proportion by the Wilson-Score method (Wilson 1927). Two true proportions were considered significantly different when their confidence interval did not overlap. To analyze the total number of eggs or egg clusters laid by each female and egg viability, negative binomial distribution was assumed, by adding all the eggs obtained from each pair up to the death of one of them, with *natural logarithm* as link function. All the means of the variables studied were separated using Tukey–Kramer's test where the F-ratio was significant ($P < 0.05$).

EPG assessments

Adult females of *P. guildinii* were obtained from an established colony initiated from adults collected from the fields of the Embrapa Wheat Experiment Station at Passo Fundo, RS, Brazil (52°24'W, 28°15'S). The laboratory colony of stink bugs was reared under controlled conditions (26±1ºC, 65±5% RH, 14:10 L:D) into plastic boxes (20 x 25 cm) lined with filter paper and fed with green pods of *Phaseolus vulgaris* (L.), raw shelled peanut *Arachis hypogaea* (L.), and pre-hydrated grains of non-Bt soybean and water.

The same two soybean varieties evaluated for the biological assessment were used to evaluate the feeding behavior of female adults of *P. guildinii*: DM59i (RR) and DM5958iPRO (RR/Bt INTACTA^{TM}). Soybean seeds were sown into small plastic pots (100 ml) filled with sterilized soil and maintained in a greenhouse. Both varieties received identical agronomic management, guaranteeing their requirements of soil humidity and nutrition and keeping them free of pests. Plants at the R6 stage (Fehr and Caviness 1977) were used for EPG studies. The plant electrode was introduced into the wet sand.

Assessment was performed by comparing the behavior of adult female of *P. guildinii* feeding on non-Bt or Bt plants. To record their feeding waveforms, a third-generation EPG was used with four channels and an AC-DC monitor (Backus and Bennett 2009; EPG Technologies, Inc., Gainesville, FL). Adult females of *P. guildinii* were immobilized, wired in an electrode, individually connected to an EPG amplifier and placed on soybean pods of one of the two varieties evaluated (see Lucini et al. 2016 for more details). Fifteen females for each treatment were monitored undisturbed for a 15-h access. Output signals were digitized at a rate of 100 samples per channel using a DI-710 (Dataq Instruments, Akron, OH) and recorded using a HP Pentium notebook with WinDaq Lite software (Dataq). The recordings were processed with the head stage amplifiers placed inside a Faraday cage and kept in a closed room with artificial light at 25 ± 2 ºC. Waveforms were characterized using an EPG waveform library of *P. guildinii* (Lucini et al. 2016) and classified in non-probing (Np) and probing waveforms (feeding). Ingestion phases (Pg) were composed by stylet penetration deep into plant tissue (Pg1), leaves, stems and pod xylem sap (Pg2), seed salivation (Pg3a) or endosperm ingestion (Pg3b).

Statistical analysis.

The means of the different waveform parameters for each treatment (Bt and non-Bt) were analyzed. Generalized linear mixed models of the statistical package SAS On Demand for Academics version 9.04 (SAS Institute Inc., Cary, NC, USA, 2018) were adjusted for the number of waveform events per insect (NWEI) and waveform duration within the total recorded period (PRT), assuming over-dispersed Poisson distribution with *log* as link function for NWEI and over-dispersed binomial distribution with *logit* as link function for PRT. The means of these variables were separated using Tukey-Kramer's test where the F-ratio was significant ($P < 0.05$). Kruskal-Wallis's non-parametric test ($P < 0.05$) was used to analyze waveform duration per probe (WDPI) and per insect (WDI), WDPI standard deviation (WDPI SD) and WDPI coefficient of variation (WDPI CV).

Results

Cry1Ac detection

The Cry1Ac protein was detected in the totality of the evaluated sets of digestive tubes of *P. guildinii* adults feeding on Bt soybean pods for 10 days (95% confidence, Table 1, Figure A2). These results indicated that this stink bug species is able to ingest the Bt protein expressed in soybean pods. The absence of the protein was verified in specimens fed with non-Bt soybean pods (95% confidence, Table 1).

In the body tissues of *P. guildinii* adults (excluding the digestive tube, head, or extremities), Cry1Ac was not detected neither in Bt nor in non-Bt treatments (95% confidence, Table 1).

Cry1Ac was found in the fecal residues of adults feeding with Bt soybean pods and not detected in the feces of adults fed with non-Bt pods (95% confidence, Table 1).

In the egg clusters *of P. guildinii*, Cry1Ac was not detected neither in Bt nor in non-Bt treatments (95% confidence, Table 1).

Table 1. Cry1Ac detection by the ELISA test (Quickstix, EnviroLogix®) in different tissues, feces and egg clusters of *Piezodorus guildinii* adults after ten days feeding with non-Bt and Bt soybean pods. The confidence interval for the true proportion and p value were estimated by the Wilson Score method (95% confidence level).

	n value	Non-Bt[1]	Bt [1]	Confidence Interval	$\widehat{\rho}$
Digestive tube	6[2]	-	+	$[0.6 - 1]$	1
Body	5[3]	-	-	$[3.139^{E-17} - 0.4344]$	0
Feces	5[4]	-	+	$[0.5655 - 1]$	1
Egg clusters	5[5]	-	-	$[3.139^{E-17} - 0.4344]$	0

[1] detection was considered positive if the sample contained a concentration value greater than or equal to 0.3% of Cry1Ac (EnviroLogix 2013).

[2] constituted by 10 digestive tubes.

[3] constituted by the body tissues (without digestive tube, head or extremities) of 5 adults.

[4] constituted by 0.15 grams of filter paper with feces signals.

[5] constituted by 15 eggs.

Biological, reproductive and survival variables measured in *P. guildinii* fed on Bt and non-Bt soybean pods

No differences were detected between *P. guildinii* fed with Bt or non-Bt soybean pods in developmental time from third instar to adult (F=0.17, P=0.68), adult longevity (F=1.29, P=0.26), and life span (time between nymph hatching to adult death) (F=1.71, P=0.19) (Figure 1).

Stink bug survival within each immature instar did not differ between treatments (95% confidence interval was overlapped, Figure 2).

Feeding with Bt pods did not affect the fecundity, evaluated through the total number of egg clusters (F=0.16, P=0.69) and the total number of eggs (F=0.02, P=0.89) laid per female during its whole adult stage (Figure 3). Egg viability was also not affected (F=1.54, P=0.21, Figure 4).

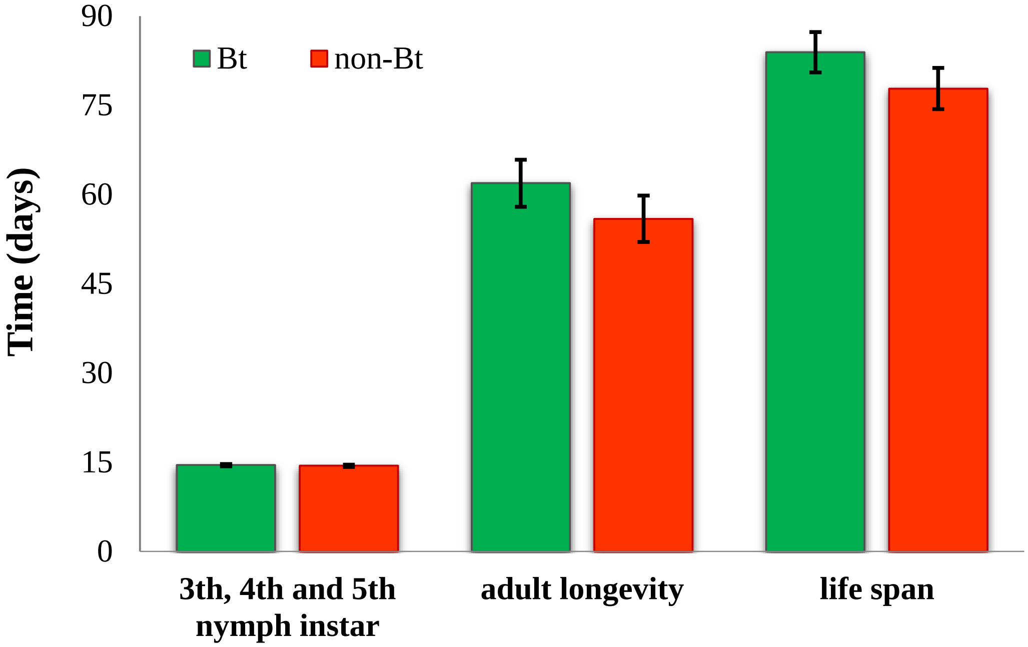

Figure 1. Developmental time from third nymphal instar to adult; adult longevity and life span (from nymph hatching to adult death) of *Piezodorus guildinii* fed with Bt and non-Bt soybean pods.

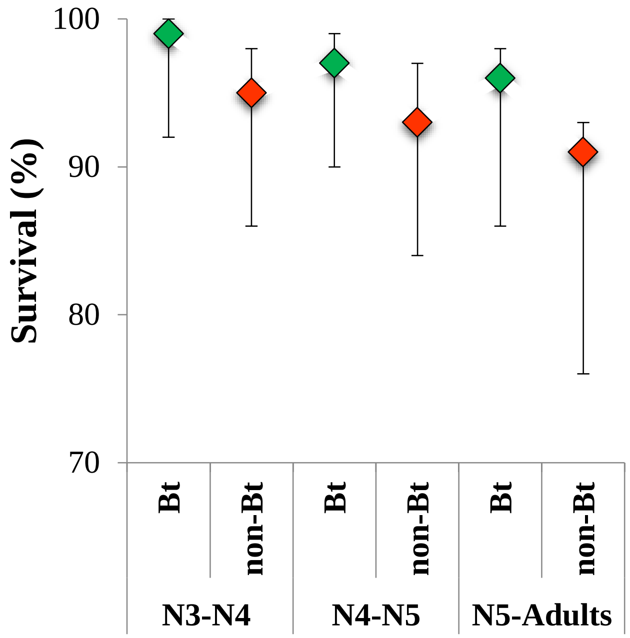

Figure 2. Percentage of survival in different immature stages of *Piezodorus guildinii* fed with Bt and non-Bt soybeans, and its confidence limits (95%, Wilson-Score method).

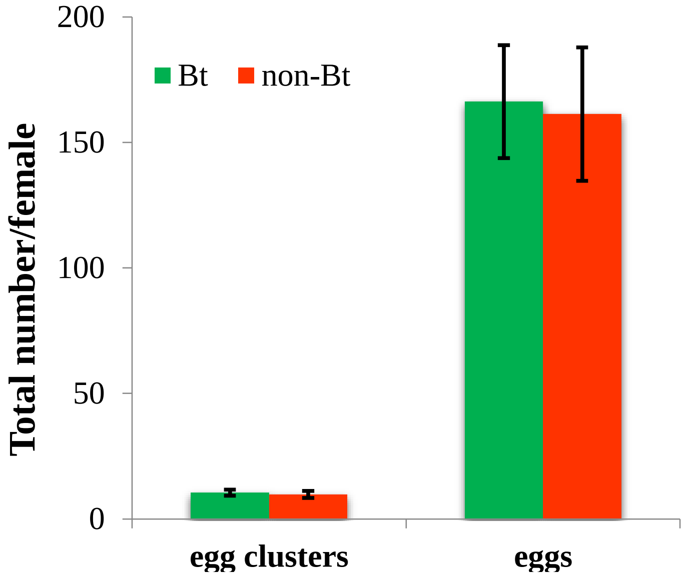

Figure 3. Total number of egg clusters and total number of eggs laid by *Piezodorus guildinii* females fed with Bt and non-Bt soybeans

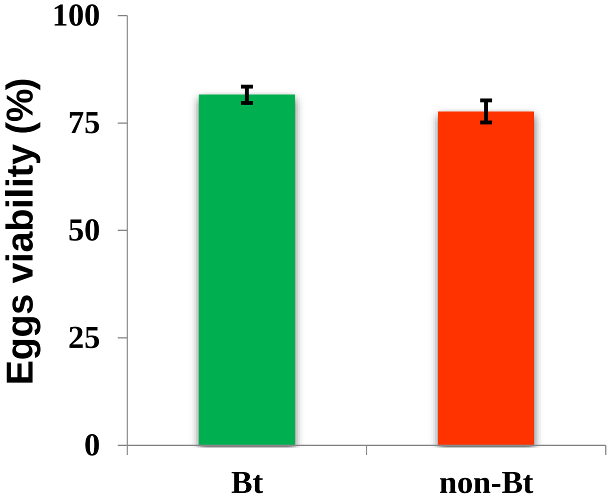

Figure 4. *Piezodorus guildinii* egg viability of mothers fed with Bt and non-Bt soybeans pods.

Feeding behavior

Different feeding behaviors between stink bugs fed with the different varieties evaluated (Bt or non-Bt) were only detected in the pathway phase Pg1 (stylet penetration deep into plant tissue) at the WDPI, WDPI SD, WDI and PRT parameters ($P < 0.05$, Table 2). Food ingestion on leaves, stems and pods, salivation on pods and non-probing phases did not differ between treatments ($P < 0.05$, Table 2).

Table 2. Means and standard errors of different EPG AC-DC waveforms of *Piezodorus guildinii* females feeding on pods of Bt or non-Bt soybean plants characterized by Lucini et al. 2016: non-probing (Np); stylet penetration deep into plant tissue (Pg1); leaves, stems and pod xylem sap (Pg2); seed salivation (Pg3a); and endosperm ingestion (Pg3b). Evaluated through number of waveform events per insect (NWEI), waveform duration within the total recorded period (PRT), waveform duration per probe (WDPI) and per insect (WDI), WDPI standard deviation (WDPI SD) and WDPI coefficient of variation (WDPI CV).

* Waveforms with p- value <0.05 differ statistically by Tukey-Kramer's test, indicated in bold type.

		PRT*			**NWEI***			**WDPI****			**WDPI SD****			**WDPI CV****			**WDI ****		
		Mean	**S.S.**	**p**	**Mean**	**S.S.**	**p**	**Mean**	**S.S.**	**p**	**Mean**	**S.S.**	**P**	**Mean**	**S.S.**	**p**	**Mean**	**S.S.**	**p**
Np	**non-Bt**	83.7	4.9	0.257	4.0	0.3	0.105	14882.9	1912.1	0.177	19848.7	3211.7	0.152	132.3	11.9	0.717	45198.2	2092.5	0.395
	Bt	75.4	4.8		3.2	0.3		11648.0	1896.2		13064.5	2330.5		119	15.0		40708.5	2913.3	
Pg1	**non-Bt**	**1.9**	**0.3**	**0.015**	5.9	0.6	0.195	**112.9**	**17.2**	**0.012**	**135.2**	**23.2**	**0.033**	117.3	8.8	0.904	**501.3**	**107.7**	**0.005**
	Bt	**0.9**	**0.2**		4.6	0.7		**177.1**	**14.9**		**218.2**	**26.5**		124.9	11.1		**1055.4**	**155.6**	
Pg2	**non-Bt**	4.4	1.3	0.464	1.9	0.3	0.758	1370.3	316.8	0.157	1437.8	375.5	0.537	111.0	19.8	0.792	2383.1	525.5	0.455
	Bt	3.2	0.9		1.7	0.4		820.6	208.2		1015.6	447.6		91.4	26.2		1761.8	567.8	
Pg3a	**non-Bt**	17.9	4.2	0.213	69.4	23.2	0.171	201.5	58.0	0.953	367.3	79.6	0.955	137.8	13.9	0.088	5471.9	1815.2	0.337
	Bt	10.1	3.9		23.7	15.9		210.6	51.4		454.4	115.9		178.5	19.1		9669.5	2440.6	
Pg3b	**non-Bt**	1.4	0.4	0.158	67.2	23.0	0.173	12.1	4.6	>0.999	24.7	11.6	>0.999	176.9	24.4	0.699	275.3	121.3	0.477
	Bt	0.5	0.3		22.2	15.5		18.4	10.2		45.7	30.9		148.5	28.1		755.0	272.2	

** Waveforms with p- value <0.05 differ statistically by Kruskall-Wallis's test, indicated in bold type.

Discussion

In the agricultural landscapes of Uruguay and the Pampas and Campos biome, the adoption of Bt soybean with resistance to caterpillars could generate changes in the dynamics of other pests and their natural enemies. *Piezodorus guildinii* is consider a primary pest of soybean in this region (Zerbino et al. 2016; Bueno et al. 2021), and although the commercially available Bt event does not control this pest, the potential uptake of Cry1Ac could lead to changes in the insects' performance and behavior that need to be evaluated. The widespread adoption of Bt cotton in certain regions of North America has probably contributed indirectly to the emergence of stink bugs as major pests because of the decrease in the use of broad-spectrum insecticides (Greene and Turnipseed 1996). Conversely, the abundance of some stink bug species has decreased in Brazil since the deployment of Bt varieties, and although the causes of this have remained unclear (Panizzi et al. 2022), it could be explained by sub-lethal effects of Cry1Ac on some biological parameters or changes in the feeding behavior of stink bugs. The results obtained in this study confirm our initial hypotheses that *P. guildinii* is able to ingest the Cry1Ac protein from Bt soybean pods without affecting its survival, development, reproduction and feeding behavior.

Cry1Ac detection

Cry1Ac was detected in the digestive tube of *P. guildinii* adults, which confirms that this stink bug is able to ingest it from Bt soybean pods. Stink bugs probably take it from the fiber cap cells of the pods and from the seed endosperm, since Cry proteins are not generally found in the vascular bundles (Raps et al. 2001; Torres et al. 2006). The ability to ingest Cry proteins was also observed in other heteropteran species, as evidenced by its presence in the digestive tube of *Lygus hesperus* (Hemiptera: Miridae) (Brandt et al. 2004) and *Dolycoris baccarum* (Hemiptera: Pentatomidae) (Yu et al. 2014), although very low or null concentrations of Bt proteins were detected in species of other families (Hemiptera: Aphididae and Fulgoridae) that feed on Bt plants (Burgio et al. 2011; Romeis and Meissle 2011; Yu et al. 2011; Zhao et al. 2013).

Cry1Ac ingestion does not necessarily determine its absorption, and even if it is absorbed, the insect could detoxify and excrete it. To exert its insecticidal action, the protein must be solubilized in an alkaline medium to break the epithelial cells and reach the hemolymph (Portela-Dussán et al. 2013). This medium is characteristic of lepidopterans guts, which have a pH of 8 to 12. In contrast, the pH of hemipterans guts varies from 4.5 to 5 (Panizzi and Parra 1991; Chapman 1998). After solubilization, the protein must be activated by proteases and subsequently recognized by specific receptors in the microvilli of the insect's midgut (Soberón and Bravo 2007). Results obtained in this study would indicate that this did not occur in *P. guildini* because the protein was not detected in its body. The absence of Cry1Ac in the body tissues could be due to the lack of the activation process, or because stink bugs do not present the specific receptors. In our study, the detection of Cry1Ac in *P. guildinii* feces would confirm that it is ingested and excreted without being transformed. Similar results were obtained in other heteropterans species (Brandt et al. 2004; Yu et al., 2014).

The ELISA detection test allows detecting the presence of the protein when it is found in concentrations greater than or equal to 0.3 % of the sample (EnviroLogix 2013). In contrast with detection test, the quantification ELISA test may be a better alternative because it has a detection limit of 0.02 µg/g dry weight, which implies a higher detection sensitivity (0.000002%). In addition, the adoption of this type of test could determine the presence of Cry1Ac in soybean Bt pods used to feed the stink bugs, without knowing the exact concentration at which it was expressing. Since it is known that the expression levels of cry proteins vary among plant tissues, developmental stages and environmental conditions (Raps et al. 2001; Lorch and Then 2007; Nguyen and Jehle 2007), it would be preferable to know the exact concentration expressed by the evaluated pods.

Biological and reproductive *P. guildinii* parameters

If *P. guildinii* is not able to absorb the protein, the possible side effects on its fitness would be almost nil. However, due to the aforementioned limitations in the sensitivity of the detection test used, biological assessments were required to complement the information obtained. As it was expected, there were no significant differences in nymphal survival between individuals feeding on Bt or non-Bt soybean pods, as it was already reported that Cry proteins are not effective to control hemipteran insects

(Chougule et al. 2013). However, this does not necessarily mean the absence of sub-lethal effects. The potential alterations in any of the biological or reproductive parameters of an important pest species such as *P. guildinii* could determine changes in its population dynamics and their respective natural controllers. Our results show no significant differences in the developmental time (from N3 to adult), adult longevity, adult fecundity and egg viability of *P. guildini*, suggesting no sub-lethal effects. There is an exhaustive bibliography related to the side effects of Cry1Ab expressed in maize on non-target pest, mainly on aphids. No deleterious effects were reported on the majority of them (Dutton et al. 2002; Ramirez-Romero et al. 2008), although aphid population and biological responses to Bt crops would depend on the aphid form and species considered and on the Bt event studied (Ramirez-Romero et al. 2008). Lumbierres et al. (2004) reported changes in the developmental time of *Rhopalosiphum padi* reared on Bt-maize plants (compared with non-Bt plants), which varied between alate or apterous aphid forms. In contrast, positive effects on development and reproduction were also reported when the predator *Orius majusculus* (Hemiptera: Anthocoridae) was reared on preys feeding on Bt maize plants (Lumbierres et al. 2012). Among pentatomid species, the results obtained are also dissimilar. The results obtained in the present study agree with those reported by Silva et al. (2014), who determined that the developmental time, fecundity and egg viability of *Euschistus heros* (Hemiptera: Pentatomidae) were not affected by the Cry1Ac protein. In contrast, adverse effects on fecundity (Bell et al. 2003) or biology (Cunha et al. 2012; Bell et al. 2005) were recorded in certain species of predatory pentatomids when they were fed with prey reared on Bt plants.

Because *P. guildini* ingests the Cry1Ac protein and does not modify it within its digestive tract, predators of *P. guildinii* feeding on them in Bt soybeans could come into contact with the protein, which is known as the prey-mediated pathway (Romeis et al. 2004). This potential risk could be mediated by the time the protein remains inside the body. If the protein is excreted soon after intake, then the risk would be low. No data about *P. guidini*'s retention time of Cry1Ac are available and we did not study this factor. Since the protein was not detected in *P. guildinii* egg clusters, the potential risks for its main natural enemies, the egg parasitoids (Zerbino and Panizzi 2019), are also reduced. The biology and reproductive parameters of the egg parasitoid *Telenomus podisi* (Hymenoptera: Platygastridae) were not affected when it was reared on eggs laid by *E. heros* feeding on Bt soybean, expressing the Cry1Ac protein (Silva et al. 2014).

Feeding behavior

The feeding behavior of non-target species of Bt technology could also be affected as a consequence of the transgenic protein expression. The EPG assessment of our study determined that the feeding behavior of *P. guildinii* adults feeding on Bt or non-Bt soybean pods of whole plants only differed within the ingestion, in the pathway phase. On the other hand, food ingestion on leaves, stems and pods, salivation on pods and non-probing phases did not differ between adults feeding on the different varieties. Studies in cotton (Olson et al. 2011) and soybean (Schünemann et al. 2018) concluded that phytophagous stink bugs did not show preferences for Bt varieties, although it was not determined whether there were modifications in the feeding process itself. In a field study, aphid density on Bt maize was higher than the conventional isogenic hybrid (Pons et al. 2005), but the probable reasons proposed did not considered changes in the feeding behavior. The active-toxin form of the Cry1Ac protein did not elicit a feeding deterrent response in *L.hesperus* (Hemiptera: Miridae) feeding on an artificial diet (Brandt et al. 2004).

Differences found during the pathway phase, when the insect stylet penetrates deep into the plant tissue, could be attributed to morphological differences between varieties and not to an effect attributable to the expression of the Cry1Ac protein. The two soybean varieties assessed were near-isogenic lines, but not exactly the same, because we wanted to evaluate commercial genotypes and there are no commercial isolines with and without the event MON 87701 (conferring the Cry1Ac expression).

The assessment of this study is the first to evaluate the potential side effects of the Cry1Ac protein expressed in Bt soybean pods on *P. guildinii*. In addition to mortality, we evaluated sub-lethal effects, which are not always detectable under field conditions. Given the high exposure of this species to Cry1Ac and its economic importance, possible modifications in its fitness and feeding behavior could have major impacts on the agricultural landscapes of the region. Our results suggest that, although *P. guildinii* ingests the Cry1Ac protein, there is no direct effect on mortality, developmental time, reproductive performance and feeding behavior traits. This is reinforced by field studies in which changes in stink bug densities in soybean crops were not reported (Abbate et al. 2022b; Abbate et al 2022c). Based on this evidence, we can conclude that the agricultural practices to control stink bugs in soybean should not differ between Bt and not-Bt varieties.

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5.1. DISCUSIÓN GENERAL

5.1.1. Factores de paisaje y locales que afectan la dinámica de poblaciones de insectos plaga y artrópodos predadores en el marco del manejo integrado

El concepto de manejo integrado de plagas (MIP) surgió a principios de 1970 en respuesta a las preocupaciones sobre los impactos de los fitosanitarios en el medio ambiente (Nicholls, 2010). El MIP se centra en el estudio de la ecología de una especie plaga, la identificación de su nicho ecológico y su interacción con otros organismos y con el medio ambiente, con el fin de comprender la dinámica de la población (Romero, 2004). Sin embargo, el nivel de operación de la mayoría de los programas de MIP implementados y adoptados en diversas regiones del mundo está basado a escala de cultivo (Kogan y Shenk, 2002). Para lograr un manejo sustentable de plagas en los cultivos extensivos, el MIP debe integrar diversas escalas de análisis; en este sentido, la información generada en esta tesis contribuye al conocimiento de factores de paisaje (composición de la cobertura vegetal) y locales (tecnología Bt, márgenes de chacra y áreas refugio), que afectan la dinámica de población de las principales especies plaga y predadores del cultivo de soja en estudios de campo. Además, se ahonda en el conocimiento, en condiciones controladas, del posible impacto de la proteína Bt en una de las principales plagas del cultivo de soja no diana de dicha tecnología: *Piezodorus guildinii*. Conocer y comprender los factores locales y de paisaje que afectan la dinámica de insectos plaga y sus controladores naturales resulta primordial para desarrollar estrategias de MIP en soja. Se espera que los resultados obtenidos contribuyan al diseño de prácticas agronómicas que maximicen la biodiversidad y el control biológico natural, procuren una adopción responsable de las variedades Bt y minimicen el uso de insecticidas. Asimismo, el conocimiento generado aportará al diseño de estrategias de intensificación ecológica (González et al., 2022, Bommarco et al., 2013) en los sistemas agropecuarios de producción de soja nacionales y regionales, en los cuales se maximicen los servicios ecosistémicos.

5.1.1.1. La composición de la cobertura vegetal como factor del paisaje determinante de la abundancia y riqueza de artrópodos predadores e insectos plaga

Los agroecosistemas de Uruguay y de América del Sur han sufrido la simplificación de los paisajes productivos debido a la adopción de la agricultura continua, principalmente asociada a la siembra de soja (Gazzano et al., 2019, Modernel et al., 2016) y en el bioma Pampas y Campos también asociada al desplazamiento de pastizales naturales (Baeza y Paruelo, 2020, Garaycochea et al., 2020). Al igual que en el resto del mundo, este proceso de intensificación agrícola determinó la remoción de hábitats menos perturbados y el incremento del uso de insecticidas, lo que causó, entre otras, la pérdida de biodiversidad y afectó negativamente a los controladores naturales (Tscharntke et al., 2021, Altieri y Nicholls, 2020, Tiscornia et al., 2014, Geiger et al., 2010). Entre éstos, los artrópodos predadores cumplen un rol ecosistémico fundamental brindando control biológico natural (Torres et al., 2018, Pons et al., 2009, Costanza et al., 1997), con potencial de mantener en niveles razonablemente bajos las poblaciones de numerosas insectos y ácaros fitófagos (Naranjo et al., 2015, Pons et al., 2009, Zhang et al., 2007) y disminuyen la necesidad de control químico. En el cultivo de soja, las arañas, los heterópteros predadores, los coccinélidos y las crisopas resultan fundamentales en este sentido (Pompozzi et al., 2021, Ribeiro, 2010), mantienen, en muchos casos, la densidad de plagas secundarias por debajo de los umbrales de acción tanto en soja como en otros cultivos que comparten los mismos enemigos naturales (Ribeiro, 2010).

Los insectos fitófagos y sus enemigos naturales responden a la composición y disposición espacial de la cobertura del paisaje circundante (Woltz et al., 2012, Gardiner et al., 2009, Tscharntke et al., 2005). La abundancia y diversidad de los enemigos naturales es afectada negativamente por la intensificación agrícola (Bianchi et al., 2006, Tscharntke et al., 2005), ya que las especies benéficas pueden requerir recursos bastante específicos en diferentes momentos y escalas espaciales (Landis et al., 2005). Estos recursos son generalmente otorgados por los hábitats menos perturbados, los cuales fueron desplazados por este fenómeno, tales como los bosques nativos (González et al., 2017). Por este motivo, los enemigos naturales se ven mayoritariamente beneficiados en paisajes complejos donde los hábitats naturales o seminaturales les proporcionan los recursos alternativos necesarios (González et al., 2022, Pompozzi et al., 2021, Chaplin-Kramer et al., 2011, Landis et al., 2000). Además, en muchos casos, las áreas menos perturbadas proporcionan el *pool* de artrópodos benéficos que recolonizan el cultivo (Madeira et al., 2019) y establecen el nivel de control biológico (Blitzer et al., 2012, Lee et al., 2001).

La composición del paisaje también juega un rol de relevancia en la determinación de los niveles de insectos fitófagos en los campos agrícolas (Tscharntke et al., 2005, Samaranayake y Costamagna, 2018). En contraposición de lo que ocurre con los enemigos naturales, generalmente la abundancia de especies fitófagas se ve favorecida en paisajes simples con predominio de áreas cultivadas (Altieri y Nicholls, 2020, Veres et al., 2013). Estas especies son más propensas a encontrar y permanecer en parches más densos y menos diversos de sus plantas hospederas, tal como lo plantea la hipótesis de concentración de recursos (Root, 1973) constatada en diversos trabajos (González et al., 2017, Nicholls, 2010, Andow, 1991, Kareiva, 1983). Se propone también que el número de insectos fitófagos es menor en los paisajes complejos debido a que los depredadores y parasitoides son más eficaces en estas condiciones, lo que se asocia a la hipótesis de enemigos naturales (Root, 1973), asociado, en ciertos casos, un mayor nivel de control biológico natural (Gardiner et al., 2009, Bianchi et al., 2006).

En cultivos de soja, se ha observado que las poblaciones de áfidos se ven favorecidas en paisajes con mayor intensificación agrícola (Maisonhaute et al., 2017, Noma et al, 2010) y que el control natural ejercido por predadores generalistas aumenta en cultivos rodeados por cobertura más diversa (Gardiner et al., 2009), dado que los predadores colonizan el cultivo desde hábitats adyacentes (Samaranayake y Costamagna, 2018). Sin embargo, la complejidad del paisaje no siempre implica mayor control biológico (Kheirodin et al., 2022, Karp et al., 2018, Tscharntke et al., 2016). Asimismo, ciertas especies fitófagas y polífagas pueden ser favorecidas en paisajes complejos, como es el caso de las chinches que pueden alimentarse de un amplio rango de hospederos cultivados y silvestres (Zerbino et al., 2012, 2014, Panizzi, 1997), lo que facilita su dispersión entre cultivos y hábitats diferentes (Venugopal et al., 2014, Tillman et al., 2009). Para este grupo de insectos plaga, la coexistencia de soja con praderas plurianuales de fabáceas en paisajes complejos representa una fuente continua de alimento. Inicialmente, las chinches se alimentan de semillas y frutos inmaduros de leguminosas y colonizan el cultivo de soja cuando comienzan a formarse los granos (Miguel et al., 2018, Zerbino et al., 2015, Ribeiro y Castiglioni, 2009, Ribeiro et al., 2009).

Los resultados obtenidos en el segundo capítulo de esta tesis (artículo 1, Abbate et al., 2022b) nos permiten afirmar que, a escala paisaje, la composición de la cobertura vegetal circundante (1 km) del cultivo de soja determina cambios en la abundancia de las principales especies de insectos plaga y artrópodos predadores presentes en el cultivo. Además, se corroboró que estas respuestas son modificadas por variables temporales como la fenología del cultivo, tal como fue previamente reportado (Kheirodin et al., 2022, Madeira et al., 2022, González et al., 2016; Ribeiro, 2007). Sin embargo, los resultados obtenidos confirman solo parcialmente la hipótesis inicialmente planteada (1a): los cultivos de soja inmersos en paisajes con predominio de cultivos anuales presentan mayor abundancia de plagas y menor abundancia de predadores. En concordancia con la hipótesis, la proporción de campo natural en el paisaje se asoció positivamente con la abundancia de predadores (arañas, hemípteros y coleópteros) y la abundancia de arañas, crisopas y hemípteros se asoció negativamente con la proporción del área de soja. Contrariamente, el porcentaje de ciertos cultivos anuales fue asociado positivamente con algunos grupos benéficos, como, por ejemplo, entre los coccinélidos y la superficie con sorgo o las arañas y la superficie de maíz. En el caso de los insectos plaga evaluados, no se constató un incremento de su abundancia en paisajes con predominio de cultivos anuales. La abundancia de chinches fitófagas se asoció positivamente con determinado componente de la cobertura vegetal, el bosque nativo, probablemente debido a características propias de la bioecología asociadas a sitios preferidos para hibernar (Zerbino et al., 2020).

Los resultados obtenidos en el marco del segundo capítulo de esta tesis corroboran que no todas las especies responden de la misma manera frente a cambios a escala de paisaje, tal como lo señalaron trabajos precedentes (Rossetti et al., 2014, Barberi et al., 2010, Attwood et al., 2008). Las diversas asociaciones detectadas entre la abundancia de diferentes grupos de artrópodos evaluados (algunas positivas y otras negativas) explican los resultados obtenidos en el marco del segundo artículo. En este se determinó que la abundancia y riqueza de artrópodos predadores en chacras de soja Bt no presentaba diferencias en paisajes caracterizados por los índices de diversidad de la cobertura vegetal circundante evaluados (riqueza y uniformidad). Estos resultados constatarían que la presencia de ciertas especies vegetales presenta mayor efecto sobre determinadas especies de artrópodos que la diversidad taxonómica de la cobertura vegetal en su conjunto, tal como señalaron Koricheva et al. (2000). Por este motivo, los cambios en las comunidades de artrópodos responderían principalmente a los cambios en la composición de la comunidad vegetal y no a la riqueza vegetal global (Haddad et al., 2001). En concordancia, también los niveles de predación natural en los agroecosistemas se relacionan mejor con la proporción de ciertos componentes de la cobertura vegetal que con la diversidad general de cultivos en el paisaje (Kheirodin et al., 2022).

5.1.1.2. Factores locales determinantes de la abundancia de artrópodos predadores e insectos plaga: Vegetación espontánea de los márgenes de chacra

Los parches de vegetación espontánea ubicados en los márgenes de chacra resultan de crucial importancia en los paisajes que han sido simplificados debido al fenómeno de intensificación agrícola, en tanto son sitios que se mantienen perturbados en menor medida por dicho fenómeno. El rol ecológico de los márgenes ha sido reportado en diversas regiones y sistemas productivos (Clemente-Orta et al., 2020, Martin et al., 2019, Mkenda et al., 2019, Thies y Tscharntke, 1999), pero poco explorado en el bioma Pampas y Campos, profundamente afectado por la agriculturización. La diversidad vegetal en estas áreas conllevaría a una mayor diversidad de predadores y parasitoides (Altieri y Nicholls, 2004), ante la homogeneización del paisaje determinada por la expansión de los cultivos anuales. Los márgenes de chacra auspician de refugio para las especies benéficas ante actividades agrícolas adversas (aplicación de insecticidas), como sitios de hibernación o proporcionando fuentes de alimentos alternativos en momentos específicos, para recolonizar el cultivo posteriormente (Pompozzi et al., 2021, Martin et al., 2019, Madeira y Pons, 2016, Blitzer et al., 2012, Thies y Tscharntke, 1999, Altieri y Whitcomb, 1979). Los márgenes de chacra también resultan de gran importancia en el momento de la cosecha de los cultivos, ya que muchas especies predadoras, como las arañas, se hospedan allí hasta la emergencia del próximo cultivo (Liljesthrom et al., 2002) y, además, utilizan estos hábitats para reproducirse (Pompozzi et al., 2021). El movimiento de los enemigos naturales desde y hacia los márgenes del cultivo es acompañado, en muchos casos, del incremento de los niveles de control natural de plagas (Madeira y Pons, 2016, Thies y Tscharntke, 1999, Altieri y Nicholls, 2004).

La vegetación circundante de los márgenes de chacra también determina la dinámica de dispersión de insectos fitófagos, tales como las chinches, que utilizan estos parches de vegetación nativa para colonizar el cultivo de soja (Zerbino et al., 2012, Ribeiro y Castiglioni, 2009, Panizzi, 1997) a pesar de que estos no ofrezcan una fuente de alimentación adecuada (Ribeiro et al., 2009, Liljesthrom y Coviella, 1999). La abundancia de especies fitófagas en los márgenes de chacra estaría asociada a la composición botánica de estos (Asutin y Brehm, 2019) y en caso de carecer de los hospederos adecuados, la permanencia en éstos sería temporal.

En el marco de los estudios realizados para responder el primer objetivo específico (capítulo 2), se estudió el rol funcional de la vegetación espontánea de los márgenes de chacra como factor local determinante de la abundancia de artrópodos en el cultivo de soja. Los resultados obtenidos permitieron corroborar la hipótesis planteada: la abundancia de predadores en la vegetación espontánea de los márgenes se asocia positivamente con la abundancia de estos dentro del cultivo. Esta relación se produjo aun en los casos en los cuales la abundancia de predadores fue mayor dentro del cultivo en comparación con el valor registrado en los márgenes (por ejemplo, en el caso de los hemípteros predadores). Los resultados también indican que los predadores presentan una respuesta numérica, es decir que es afectada por la abundancia respectiva de sus presas, las cuales son más abundantes generalmente dentro del cultivo. Esta respuesta ha sido reportada en otros trabajos (Madeira et al., 2022, Meseguer et al., 2021, Pons et al., 2005a). Los márgenes auspiciarían de refugio en determinados momentos. Además, en el caso de insectos plaga, no se obtuvieron asociaciones entre la abundancia en los márgenes y dentro del cultivo, lo que permite sugerir que estos parches de vegetación espontánea que limitan las chacras de soja son importantes fuentes de predadores sin implicar un riesgo mayor en el caso de los insectos fitófagos. Por este motivo, se resalta la importancia de incluir prácticas agronómicas que tiendan a preservar la vegetación espontánea de los márgenes dentro del enfoque de sustentabilidad en el asesoramiento técnico.

5.1.1.3. Factores locales determinantes de la abundancia de artrópodos predadores e insectos plaga: Variedades transgénicas resistentes a insectos plaga (tecnología Bt)

La incorporación de cultivos genéticamente modificados en los sistemas de producción agrícola resulta en un factor local que, en el caso de las variedades Bt (resistentes a insectos plaga), repercute en el manejo de plagas, ya que implica cambios en la dinámica de población de los insectos fitófagos. Si bien la incorporación de cultivos Bt permite el control específico de plagas, reducen la necesidad de aplicación de insecticidas de amplio espectro (Pellegrino et al., 2018, Brookes y Barfoot, 2006) y disminuyen su efecto sobre los enemigos naturales (Romeis et al., 2019, Romeis et al., 2006), existen riesgos potenciales que deben ser estudiados (Groot y Dicke, 2002, Hails, 2002). Las posibilidades de generar efectos no deseados sobre organismos no diana son elevadas debido a la expresión continua de las toxinas Bt en las diferentes estructuras de la planta durante todo el ciclo de crecimiento (Williamson, 1992).

Entre los organismos no diana de la tecnología Bt se encuentran los artrópodos predadores, los cuales podrían entrar en contacto con las toxinas Bt de manera directa a través del polen o del tejido vegetal que muchos de ellos ingieren como alimento suplementario a sus presas (Torres et al., 2006). También podrían exponerse directamente a estas mediante el consumo de presas alimentadas directamente en plantas Bt, siendo mayor la probabilidad en fitófagos en los cuales dichas toxinas no provocan la muerte (Lumbierres et al., 2012, Borsani et al., 2010). Si bien en algunos estudios se evidenciaron efectos adversos sobre determinadas especies benéficas (Lövei y Arpaia, 2005, Pellegrino et al. 2018), la mayoría de las investigaciones de campo y laboratorio concluyen que estos no son afectados negativamente por esta tecnología (Pellegrino et al., 2018, Lumbierres et al., 2011, 2012, Sisterson et al., 2007, De la Poza et al., 2005, Pons y Starý, 2003).

Los cultivos Bt podrían también afectar a los enemigos naturales de manera indirecta, debido a la menor abundancia de sus respectivas presas (Naranjo, 2005, Zwahlen y Andow, 2005) determinada por la elevada eficacia de control de los mismos. Si bien numerosos trabajos indican que la fauna benéfica resulta más abundante en cultivos Bt en comparación con cultivos convencionales manejados con insecticidas de amplio espectro (Marvier et al., 2007, Fontes et al., 2002), el cambio a escala local generado por la adopción de la tecnología Bt debería ser evaluado teniendo en cuenta las particularidades de los sistemas productivos nacionales. Por este motivo, se planteó en la presente tesis la siguiente hipótesis: los cultivos de soja Bt presentan menor abundancia de lepidópteros defoliadores, lo que afecta negativamente la abundancia y riqueza de predadores en comparación con las chacras de soja no Bt. Refutando lo propuesto, los resultados obtenidos (Abbate et al., 2022b, Abbate et al., 2022c) indican que a pesar de que las chacras de soja Bt presentan menor abundancia de lepidópteros defoliadores, esto no implica que estas presenten menor abundancia o riqueza de artrópodos predadores. Estos resultados podrían explicarse porque la mayoría de las especies predadoras presentes en soja en Uruguay presentan hábitos alimenticios generalistas y ante la falta de sus presas preferidas se alimentarían de otros insectos presentes en el cultivo, tales como los trips y las arañuelas (Abbate et al., 2022b). Cabe aclarar que estos resultados no deberían extrapolarse hacia otros grupos de enemigos naturales, como por ejemplo los parasitoides. En este sentido, particularmente en el caso de los parasioides de huevos o de larvas de lepidópteros, con un hábito alimenticio mucho más restringido, es probable que su abundancia se vea afectada negativamente en las chacras de soja Bt, con densidades de huéspedes prácticamente nulas.

Los insectos fitófagos también se encuentran entre los organismos no blanco de la tecnología Bt que podrían ser afectados por esta, lo cual podría implicar modificaciones en la dinámica de plagas que deben considerarse dentro de los programas de MIP. Un ejemplo fue el caso de la adopción generalizada de algodón Bt en diversas regiones del mundo. La siembra de variedades de algodón Bt implicó la disminución de la aplicación de insecticidas de amplio espectro usados en dicho cultivo para el control de lepidópteros plaga, pero que indirectamente controlaban también otras plagas secundarias como las chinches. Ante la reducción de las aplicaciones de insecticidas, las poblaciones de chinches aumentaron considerablemente al igual que los daños y las pérdidas económicas provocadas por aquellas (Olson et al., 2011, Greene et al., 1999, Greene y Turnipseed, 1996). Hasta la fecha, en los estudios realizados en Uruguay a escala de chacra no se han registrado efectos negativos del maíz Bt (Cry1Ab) sobre las poblaciones de fitófagos no diana (Zerbino, 2012, Castiglioni et al., 2009, 2007, 2006, Chiaravalle y Aznárez, 2008). Dado que la soja Bt controla un rango mayor de insectos en relación con el maíz y que el área que ocupa es considerablemente superior, la menor abundancia de especies diana podría provocar importantes cambios en la comunidad de insectos fitófagos, como las chinches. Estudios previos determinaron que la abundancia de este grupo de fitófagos no difería entre chacras comerciales de algodón Bt y no Bt (Greene et al., 2001). En cambio, en maíz, la abundancia de otros grupos de hemípteros (áfidos y cicadélidos) fue mayor en variedades Bt en relación con las no Bt (Faria et al., 2007, Pons et al., 2005b, Lumbierres et al., 2004). Los resultados obtenidos en esta tesis en soja en condiciones de campo (capítulos 2 y 3) permiten afirmar que la inclusión de soja Bt en los sistemas productivos de Uruguay no determinó cambios en la abundancia del complejo de chinches (Abbate et al., 2022b, Abbate et al., 2022c).

Resulta de importancia considerar que los efectos no deseados de las proteínas transgénicas podrían incluir, además de la mortalidad, consecuencias subletales sobre los organismos no diana. Por este motivo, para ampliar las bases del conocimiento, resulta fundamental que los ensayos de campo se complementen con estudios en condiciones controladas. Si bien los insectos del orden Hemiptera presentan un aparato bucal picosuctor y no podrían ingerir las endoproteínas cry expresadas en los cultivos Bt desde los tejidos conductores del xilema y floema al no expresarse las toxinas Cry en ellos (Torres et al., 2006, Raps et al., 2001), sí podrían adquirirlos si estos se expresan en las células del mesófilo (Dutton et al., 2004). Efectivamente, ciertos efectos adversos de las toxinas transgénicas han sido registrados en algunas especies de hemípteros (Li et al., 2011, Brandt et al., 2004). En áfidos y fulgóridos, se detectaron muy bajas o nulas concentraciones de toxina al ser alimentados con plantas Bt (Burgio et al., 2011, Romeis y Meissle, 2011, Yu et al., 2011, Zhao et al., 2013). En estudios de laboratorio, la proteína Cry1Ac expresada en soja no afectó el desarrollo ni la reproducción del fitófago *Euchistus heros* (Hemiptera: Pentatomidae) (Silva et al., 2014) pero se desconoce si dicho pentatómido no adquiere la proteína de la planta o si esta no resulta tóxica a pesar de ser ingerida. Por el contrario, en ciertas especies de pentatómidos predadores se registraron efectos adversos sobre la fecundidad (Bell et al., 2003) o la biología (Cunha et al., 2012, Bell et al., 2005), cuando estas eran alimentadas con presas criadas sobre plantas Bt.

El comportamiento alimenticio de especies no diana de la tecnología Bt también podría ser afectado como consecuencia de la expresión de las toxinas. Estudios realizados en algodón (Olson et al., 2011) y soja (Schünemann et al., 2018) concluyeron que las chinches fitófagas no presentaban preferencias por las variedades Bt, aunque no se determinó si existían modificaciones en el proceso de alimentación en sí. La posible alteración del comportamiento alimenticio en hemípteros debido a la ingesta de proteínas Bt ha sido escasamente estudiado, ya que estos insectos presentan piezas bucales picosuctoras que dificultan dicha evaluación (Schünemann et al., 2014). La adaptación de técnicas de gráfico de penetración eléctrica (EPG) (Tjallingii, 1978) al estudio de la conducta alimenticia de los pentatómidos (Lucini y Panizzi, 2017a) ha permitido avances significativos en este sentido. En esta técnica se establece un circuito eléctrico simple, compuesto por la planta y el insecto que se alimenta, lo que permite la identificación de formas de onda computacionales asociadas a actividades de sondeo y no sondeo (Lucini y Panizzi, 2017b, 2016, Lucini et al., 2016). En los estudios enmarcados en el cuarto capítulo de esta tesis, se realizaron ensayos en condiciones controladas de laboratorio y se utilizó la técnica de EPG para estudiar el posible efecto de la proteína Cry1Ac sobre parámetros biológicos, reproductivos y el comportamiento alimenticio de *P. guildinii*, especie de chinche más frecuente y con mayor potencial de daño en el cultivo de soja de Uruguay (Abbate et al., 2022a). Los resultados obtenidos permitieron aceptar la hipótesis planteada: el comportamiento alimenticio, la biología y la reproducción de *P. guildinii* (especie plaga no diana de la tecnología Bt) no se ven afectados por el consumo de vainas de soja que expresan la proteína Cry1Ac; por lo tanto, el manejo de esta plaga no debería diferir entre cultivos de soja Bt y noBt.

Resulta importante destacar el hecho que en el marco de esta tesis, la tecnología Bt fue estudiada como un factor local y los resultados obtenidos deben ser interpretados desde esta escala. Sería de gran interés que líneas futuras aborden el estudio de posibles impactos de la adopción de la tecnología Bt sobre la abundancia y diversidad de diversos grupos de artrópodos (benéficos y fitófagos) como un factor del paisaje, es decir identificando dentro de la cobertura vegetal circundante, cuál es la proporción ocupada por cultivos Bt.

5.1.1.4. Factores locales determinantes de la abundancia de artrópodos predadores e insectos plaga: Áreas refugio de cultivos Bt

En el caso del cultivo de soja en Uruguay, la adopción de variedades Bt significó una táctica eficaz para reducir las aplicaciones químicas dirigidas al control de las especies de lepidópteros defoliadores más frecuentes (*Anticarsia gemmatalis, Rachiplusia nu* y *Pseudoplusia includes*). Sin embargo, dicha adopción puede suponer riesgos a largo plazo si no es incorporada como una táctica más dentro del MIP abordado a escala paisaje. El surgimiento de poblaciones resistentes es una de las mayores preocupaciones (Lombardo et al., 2020, Tabashnik et al., 2013), dado que en la actualidad ya se ha reportado la pérdida de susceptibilidad de ciertas especies a la proteína Cry1Ac en Brasil y Argentina (Vera et al., 2022, Horikoshi et al., 2021). Por este motivo, se ha propuesto implementar diferentes estrategias para retrasar el surgimiento de poblaciones resistentes enmarcados dentro del programa de manejo de la resistencia (MRI); es el caso de la utilización de la llamada alta dosis/refugio (Zhou et al., 2017, Shelton et al., 2000) y del uso de variedades piramidales que expresan diferentes toxinas Bt (Carrière et al., 2015). En el cultivo de la soja en Uruguay, dado que hasta la fecha solo se comercializan variedades Bt portadoras de un solo evento transgénico, las estrategias de MRI se basan solamente en la estrategia alta dosis/refugio. Dicha estrategia consiste en utilizar variedades Bt que expresan altas dosis de toxina que matan a la mayoría de los individuos de la plaga objetivo y asignar una determinada proporción del área sembrada con variedades no Bt, que actuarán como refugios para los individuos susceptibles (Carrière et al., 2012, Huang et al., 2011). Además, deben cumplirse algunas condiciones, entre las cuales se establecen determinados valores de tamaño y ubicación de los refugios que varían según el cultivo Bt y país; esto es esencial para que el enfoque sea eficaz (Bates et al., 2005, Bourguet et al., 2005).

Subyace otra preocupación asociada a la adopción de grandes superficies de cultivos Bt, con una elevada eficacia del control de los insectos plaga objetivo de la tecnología. La menor abundancia de presas en cultivos Bt también podría determinar una menor abundancia de enemigos naturales (Naranjo, 2005, Zwahlen y Andow, 2005). Si bien la fauna benéfica resulta más abundante en cultivos Bt en comparación con cultivos convencionales manejados con insecticidas de amplio espectro (Marvier et al., 2007, Fontes et al*.,* 2002), la falta de presas en ciertos casos podría impactar negativamente sobre los predadores, tal como fue mencionado anteriormente en este documento. En cambio, los refugios presentan una mayor cantidad de alimento para los enemigos naturales, debido a que son áreas sembradas con variedades no Bt y manejadas con base en los umbrales de daño económico, de acuerdo a los cuales se toleran ciertas densidades de insectos, y, en el caso de que se requiera la aplicación de insecticidas, estos nunca alcanzan una eficacia completa del control (Bueno et al., 2011, Ecket et al., 1993, Follett et al., 1994). En este sentido, las áreas refugio (variedades no Bt) de la tecnología Bt en soja, además de ser una estrategia para retrasar la aparición de poblaciones resistentes, cumplirían un rol fundamental garantizando un mínimo de presas para mantener las poblaciones de biocontroladores que se alimentan de estas (White y Andow, 2003), siendo este un rol ecológico de los refugios escasamente estudiado. Los resultados reportados en el segundo artículo enmarcado en esta tesis nos permitieron confirmar la hipótesis planteada: las áreas refugio de la tecnología Bt, cumplen un rol funcional determinante de la abundancia y riqueza de las poblaciones de predadores en el cultivo de soja. Se trata del primer reporte internacional de esta función ecológica de las áreas refugio.

5.2. CONCLUSIONES GENERALES

Para poder llevar adelante programas de manejo de plagas desde un abordaje de intensificación ecológica y a escala paisaje, resulta clave considerar diversos aspectos, algunos de los cuales han sido planteados y estudiados en el marco de esta tesis:

1. Comprender la asociación de la dinámica de las poblaciones de artrópodos predadores e insectos plaga con la composición de la cobertura vegetal del paisaje circundante.
2. Determinar la importancia de la vegetación espontánea de los márgenes de chacra y las áreas refugio (de cultivos Bt) en la determinación de la abundancia y riqueza de artrópodos predadores.
3. Conocer el posible impacto de las toxinas incorporadas en los cultivos Bt en las poblaciones de especies benéficas y fitófagas no diana y monitorear su eficacia sobre especies objetivo.

En relación con el punto 1, los resultados obtenidos confirman, para las condiciones de Uruguay, el conocimiento generado en el ámbito internacional, el cual indica que la abundancia de las poblaciones de los principales grupos de plagas y predadores presentes en el cultivo de soja es afectada de manera diferencial por los componentes de la cobertura vegetal. En este sentido, si bien el incremento del área sembrada con el cultivo de soja en el paisaje circundante se asoció negativamente con la abundancia de varios grupos de predadores, la presencia de otros cultivos tuvo un impacto positivo, al igual que la proporción del campo natural. En el caso de los insectos plaga, el paisaje tuvo efecto en la abundancia de insectos de menor movilidad como lo son las chinches fitófagas, pero no sobre lepidópteros defoliadores, caracterizados por su elevada capacidad migratoria. Estas asociaciones diferenciales establecidas entre los diferentes componentes del paisaje y los grupos de predadores o plagas probablemente expliquen la falta de efecto cuando estas se realizaron mediante los índices de diversidad evaluados (riqueza y uniformidad), los cuales caracterizan la diversidad de la cobertura vegetal mediante valores únicos. De esta manera, nuestros resultados corroboran que cuando se combinan todos los factores del paisaje que afectan a las diferentes especies de artrópodos en un solo índice, estas se contrarrestan entre sí y no se puede detectar claramente ningún efecto. Esto podría explicarse porque la diversidad de la cubierta vegetal *per se* es menos relevante que la forma en que se constituye esta diversidad, ya que la identidad de ciertos componentes es más importante que la diversidad entre los componentes.

En relación con el punto 2, y reafirmando la importancia de identificar los componentes del paisaje que favorezcan la abundancia y diversidad de controladores naturales, los resultados obtenidos corroboran el rol de los márgenes de chacra y de las áreas refugio. En este sentido, la vegetación espontánea presente en los límites de los cultivos de soja albergó diferentes grupos de predadores, los cuales, independientemente de resultar más o menos abundantes que los valores registrados en soja, se asociaron positivamente con su abundancia en dicho cultivo. Por otro lado, en el marco de esta tesis, se ha podido afirmar, por primera vez, que las áreas refugio de soja Bt, diseñadas como una de las estrategias para retrasar el surgimiento de poblaciones resistentes, cumplen también un importante rol en la preservación de los predadores, ya que la abundancia y riqueza registradas en estas áreas se asocia positivamente con los valores registrados en el cultivo de soja Bt. Estos resultados resaltan la importancia de preservar los márgenes de chacra y sembrar refugios con el objetivo de que auspicien de sitios de preservación y fuente de predadores en el paisaje.

Por último, en relación con el punto 3, resulta importante mencionar que las modificaciones realizadas por el hombre a escala local pueden tener consecuencias no solo en ese nivel, sino también en una escala mayor. En este sentido, la incorporación de la soja Bt en Uruguay y el bioma Pampas y Campos resultó en una eficiente táctica para controlar las poblaciones de lepidópteros defoliadores en dicho cultivo, lo que redundó en la disminución del uso de insecticidas, pero con posibles impactos sobre organismos no diana, poco abordados hasta la fecha en el país. Los resultados obtenidos en esta tesis permiten afirmar que la abundancia y riqueza de los principales grupos de predadores presentes en soja no son afectadas en los cultivos de soja Bt. En relación con el posible impacto de la tecnología Bt sobre insectos fitófagos no diana, se concluye que la proteína Cry1Ac expresada en vainas de soja Bt no afecta el desarrollo, la reproducción ni el hábito alimenticio del hemíptero pentatómido *P.*

guildinii, principal plaga de soja en Uruguay. Por lo tanto, el manejo de esta plaga en cultivos de soja debe ser semejante tanto en variedades Bt como en variedades no Bt.

El abordaje a escala paisaje enriquece las posibilidades de realizar un manejo integrado de plagas en el cultivo de soja, mientras que los ensayos en condiciones controladas permiten complementar los resultados obtenidos a campo. En el marco de programas de manejo sustentable de plagas, la implementación de diferentes tácticas, como el uso de variedades Bt, debe realizarse de manera integrada. En este sentido, la siembra de áreas refugio, la preservación de la vegetación espontánea de los márgenes de chacra y la diversificación de cultivos cumplen un rol fundamental en la preservación de la fauna benéfica, lo cual maximizaría el control natural de insectos plaga, minimizando el requerimiento de insecticidas. Los resultados obtenidos aportan posibilidades de intensificación ecológica al diseño de los sistemas productivos nacionales y regionales.

5.3. PERSPECTIVAS

La ecología del paisaje resulta una disciplina clave para ampliar las bases del conocimiento en entomología aplicada con el fin de contribuir con los programas de manejo integrado de plagas en cultivos extensivos desde una óptica de intensificación ecológica. Los datos obtenidos en el marco de esta tesis constituyen uno de los primeros aportes en el ámbito nacional, y los resultados hallados dan indicio de las temáticas que se abordarán en un futuro, las cuales pueden agruparse en:

— Determinar el rol funcional en el paisaje agrícola-pastoril de ciertos componentes de la cobertura vegetal, los cuales podrían contribuir con la abundancia y diversidad de especies benéficas: cultivos de servicio (cobertura) y colza.

— Analizar los efectos del paisaje en la dinámica de las poblaciones de plagas y predadores considerando, además de la composición de la cobertura vegetal, su estructura, dado que son numerosas las evidencias en lo internacional del efecto de la disposición de los parches de vegetación en el paisaje y su conectividad y no solo el porcentaje que estos ocupan.

— Ahondar en estudios de dirección del movimiento de las especies benéficas para determinar si las áreas asociadas positivamente con su abundancia y diversidad actúan de sumidero o de fuente y determinar los momentos más relevantes para estos procesos.

— Ampliar el grupo de artrópodos benéficos a estudiar, incluyendo, además de los predadores, a especies de insectos parasitoides y polinizadores.

— Ampliar el abanico de especies plagas y cultivos a estudiar, incluyendo, además de las principales (chinches fitófagas y lepidópteros defoliadores), plagas primarias de otros cultivos (por ejemplo, *Spodoptera frugiperda* en maíz) así como también plagas secundarias o esporádicas (trips, arañuelas, hormigas cortadoras).

— Continuar evaluando la eficacia de los cultivos Bt (soja y maíz) para el control de las plagas diana, con el fin de identificar el surgimiento de poblaciones resistentes. Asimismo, mantener las evaluaciones, a diferentes escalas, del posible impacto de la expresión de las proteínas transgénicas expresadas en soja y maíz sobre organismos no diana.

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7. ANEXOS

7.1 ANEXOS CORRESPONDIENTES AL ARTÍCULO 2

Table S1 Specific information on season, year, coordinates, municipality, field area and sampling date for the sampled fields of soybean

Year	Latitude	Longitude	Department	Tech-nology	Field area (ha)	Distance Bt-Refuge (m)	Landscape Diversity				Number of Sampling					
							% annual crops	Shannon Index	Richness	Evenness	1	2	3	4	5	6
2018	32°25'41.45"S	57°56'44.13"O	Paysandú	Bt	90.3	161	67.3	0.78	4	0.5	2-Jan*	18-Jan	12-Feb	1-Mar	21-Mar	9-Apr
2018	32°25'38.38"S	57°56'39.13"O	Paysandú	Refuge	18.1						2-Jan*	18-Jan	12-Feb	1-Mar	21-Mar	9-Apr
2018	32°21'50.12"S	57°56'44.68"O	Paysandú	Bt	29.0	264	70.8	0.79	3	0.4	2-Jan*	18-Jan	12-Feb	1-Mar	21-Mar	9-Apr
2018	32°21'58.59"S	57°56'45.16"O	Paysandú	Refuge	5.8						2-Jan*	18-Jan	12-Feb	1-Mar	21-Mar	9-Apr
2018	32°23'14.39"S	58° 4'54.86"O	Paysandú	Bt	118.3	70	74.9	0.84	5	0.5	2-Jan*	18-Jan	12-Feb	1-Mar	21-Mar	9-Apr
2018	32°23'14.34"S	58° 4'57.50"O	Paysandú	Refuge	31.8						2-Jan*	18-Jan	12-Feb	1-Mar	21-Mar	9-Apr
2018	32°31'21.92"S	57°50'44.33"O	Paysandú	Bt	87.2	238	55.7	1.04	5	0.5	2-Jan*	18-Jan	12-Feb	1-Mar	21-Mar	9-Apr
2018	32°31'15.66"S	57°50'39.53"O	Paysandú	Refuge	59.8						2-Jan*	18-Jan	12-Feb	1-Mar	21-Mar	9-Apr
2018	32°31'12.77"S	57°52'9.37"O	Paysandú	Bt	142.6	245	52.2	1.10	5	0.4	2-Jan*	18-Jan	12-Feb	1-Mar	21-Mar	9-Apr
2018	32°31'6.34"S	57°52'14.87"O	Paysandú	Refuge	81.8						2-Jan*	18-Jan	12-Feb	1-Mar	21-Mar	9-Apr
2018	31°49'54.77"S	57°48'18.05"O	Paysandú	Bt	15.8	94	48.0	1.42	6	0.4	3-Jan*	19-Jan	13-Feb	2-Mar	22-Mar	10-Apr
2018	31°50'1.35"S	57°48'14.50"O	Paysandú	Refuge	3.2						3-Jan*	19-Jan	13-Feb	2-Mar	22-Mar	10-Apr
2018	31°50'17.02"S	58° 2'12.26"O	Paysandú	Bt	29.9	459	30.6	1.17	4	0.2	3-Jan*	19-Jan	13-Feb	2-Mar	22-Mar	10-Apr
2018	31°50'27.96"S	58° 2'23.24"O	Paysandú	Refuge	11.1						3-Jan*	19-Jan	13-Feb	2-Mar	22-Mar	10-Apr
2018	31°56'20.10"S	58° 0'5.58"O	Paysandú	Bt	9.1	193	64.3	0.72	3	0.3	3-Jan*	19-Jan	13-Feb	2-Mar	22-Mar	10-Apr
2018	31°56'24.97"S	58° 0'10.06"O	Paysandú	Refuge	16.0						3-Jan*	19-Jan	13-Feb	2-Mar	22-Mar	10-Apr
2018	31°59'23.99"S	58° 2'40.15"O	Paysandú	Bt	226.9	167	73.2	0.67	4	0.5	3-Jan*	19-Jan	13-Feb	2-Mar	22-Mar	10-Apr
2018	31°59'28.89"S	58° 2'43.60"O	Paysandú	Refuge	81.1						3-Jan*	19-Jan	13-Feb	2-Mar	22-Mar	10-Apr

2018	32° 1'12.87"S	57°52'18.84"O	Paysandú	Bt	61.0	108	46.3	1.17	5	0.4	3-Jan*	19-Jan	13-Feb	2-Mar	22-Mar	10-Apr
2018	32° 1'11.94"S	57°52'22.62"O	Paysandú	Refuge	26.9						3-Jan*	19-Jan	13-Feb	2-Mar	22-Mar	10-Apr
2018	32°59'5.78"S	57°39'54.15"O	Río Negro	Bt	38.8	459	90.8	0.32	3	0.6	4-Jan*	20-Jan	14-Feb	3-Mar	23-Mar	11-Apr
2018	32°59'19.72"S	57°40'0.16"O	Río Negro	Refuge	72.2						4-Jan*	20-Jan	14-Feb	3-Mar	23-Mar	11-Apr
2018	32°55'1.99"S	57°34'22.00"O	Río Negro	Bt	58.7	316	67.7	0.89	4	0.4	4-Jan*	20-Jan	14-Feb	3-Mar	23-Mar	11-Apr
2018	32°55'12.22"S	57°34'22.75"O	Río Negro	Refuge	38.2						4-Jan*	20-Jan	14-Feb	3-Mar	23-Mar	11-Apr
2018	32°55'55.29"S	57°35'50.00"O	Río Negro	Bt	3.0	131	47.6	1.11	4	0.3	4-Jan*	20-Jan	14-Feb	3-Mar	23-Mar	11-Apr
2018	32°55'59.15"S	57°35'52.25"O	Río Negro	Refuge	35.2						4-Jan*	20-Jan	14-Feb	3-Mar	23-Mar	11-Apr
2018	32°40'21.79"S	57°21'4.59"O	Río Negro	Bt	24.2	765	82.3	0.51	3	0.5	4-Jan*	20-Jan	14-Feb	3-Mar	23-Mar	11-Apr
2018	32°38'39.36"S	57°20'15.60"O	Río Negro	Refuge	38.0						4-Jan*	20-Jan	14-Feb	3-Mar	23-Mar	11-Apr
2018	32°42'13.19"S	57°31'20.85"O	Río Negro	Bt	121.0	161	45.3	1.04	3	0.1	4-Jan*	20-Jan	14-Feb	3-Mar	23-Mar	11-Apr
2018	32°42'17.44"S	57°31'16.71"O	Río Negro	Refuge	63.6						4-Jan*	20-Jan	14-Feb	3-Mar	23-Mar	11-Apr
2018	32°45'53.83"S	57°31'21.70"O	Río Negro	Bt	221.0	189	87.0	0.43	3	0.5	4-Jan*	20-Jan	14-Feb	3-Mar	23-Mar	11-Apr
2018	32°45'59.00"S	57°31'18.23"O	Río Negro	Refuge	55.5						4-Jan*	20-Jan	14-Feb	3-Mar	23-Mar	11-Apr
2019	32°30'31.06"S	58° 4'20.16"O	Río Negro	Bt	16.2	665	46.6	1.74	8	0.4	5-Jan*	24-Jan	13-Feb	8-Mar	27-Mar	12-Apr
2019	32°30'48.27"S	58° 4'35.52"O	Río Negro	Refuge	14.7						5-Jan*	24-Jan	13-Feb	8-Mar	27-Mar	12-Apr
2019	32°30'56.56"S	58° 0'21.81"O	Paysandú	Bt	18.5	515	64.7	1.21	5	0.4	5-Jan*	24-Jan	13-Feb	8-Mar	27-Mar	12-Apr
2019	32°31'6.35"S	58° 0'5.82"O	Paysandú	Refuge	21.2						5-Jan*	24-Jan	13-Feb	8-Mar	27-Mar	12-Apr
2019	32°36'6.72"S	58° 0'54.08"O	Río Negro	Bt	38.6	466	72.2	0.90	5	0.5	5-Jan*	24-Jan	13-Feb	8-Mar	27-Mar	12-Apr
2019	32°36'21.78"S	58° 0'53.10"O	Río Negro	Refuge	25.2						5-Jan*	24-Jan	13-Feb	8-Mar	27-Mar	12-Apr
2019	32°32'36.76"S	57°58'18.01"O	Paysandú	Bt	26.1	532	53.8	1.42	7	0.4	5-Jan*	24-Jan	13-Feb	8-Mar	27-Mar	12-Apr
2019	32°32'53.91"S	57°58'20.20"O	Paysandú	Refuge	5.2						5-Jan*	24-Jan	13-Feb	8-Mar	27-Mar	12-Apr
2019	32°32'43.17"S	57°54'18.69"O	Paysandú	Bt	77.0	117	77.9	0.79	5	0.6	5-Jan*	24-Jan	13-Feb	8-Mar	27-Mar	12-Apr
2019	32°32'42.36"S	57°54'23.07"O	Paysandú	Refuge	33.6						5-Jan*	24-Jan	13-Feb	8-Mar	27-Mar	12-Apr

2019	32°14'24.17"S	57°54'3.00"O	Paysandú	Bt	23.5	91	60.5	0.98	4	0.4	6-Jan*	25-Jan	14-Feb	7-Mar	28-Mar	1-Apr
2019	32°14'21.94"S	57°54'5.27"O	Paysandú	Refuge	41.9						6-Jan*	25-Jan	14-Feb	7-Mar	28-Mar	1-Apr
2019	31°58'1.22"S	58° 6'38.38"O	Paysandú	Bt	54.4	429	34.4	1.23	4	0.2	6-Jan*	25-Jan	14-Feb	7-Mar	28-Mar	16-Apr
2019	31°57'50.37"S	58° 6'48.66"O	Paysandú	Refuge	11.1						6-Jan*	25-Jan	14-Feb	7-Mar	28-Mar	16-Apr
2019	31°48'59.06"S	57°54'13.96"O	Paysandú	Bt	18.6	120	34.9	1.08	5	0.5	6-Jan*	25-Jan	14-Feb	7-Mar	28-Mar	16-Apr
2019	31°49'2.88"S	57°54'14.92"O	Paysandú	Refuge	37.3						6-Jan*	25-Jan	14-Feb	7-Mar	28-Mar	16-Apr
2019	32°55'39.54"S	57°49'22.09"O	Río Negro	Bt	38.8	122	74.0	0.78	5	0.5	4-Jan*	22-Jan	12-Feb	6-Mar	26-Mar	
2019	32°55'41.20"S	57°49'17.83"O	Río Negro	Refuge	16.0						4-Jan*	22-Jan	12-Feb	6-Mar	26-Mar	
2019	32°55'33.96"S	57°47'59.14"O	Río Negro	Bt	37.1	347	78.5	0.63	5	0.6	4-Jan*	22-Jan	12-Feb	6-Mar	26-Mar	15-Apr
2019	32°55'33.70"S	57°48'12.49"O	Río Negro	Refuge	41.7						4-Jan*	22-Jan	12-Feb	6-Mar	26-Mar	15-Apr
2019	32°42'32.45"S	57°21'51.36"O	Río Negro	Bt	103.0	103	86.2	0.42	3	0.5	4-Jan*	22-Jan	12-Feb	6-Mar	26-Mar	
2019	32°42'29.39"S	57°21'49.79"O	Río Negro	Refuge	63.3						4-Jan*	22-Jan	12-Feb	6-Mar	26-Mar	
2019	32°57'3.78"S	57°38'40.96"O	Río Negro	Bt	58.2	429	76.6	1.00	6	0.5	4-Jan*	22-Jan	12-Feb	6-Mar	26-Mar	15-Apr
2019	32°57'3.19"S	57°38'52.29"O	Río Negro	Refuge	42.2						4-Jan*	22-Jan	12-Feb	6-Mar	26-Mar	15-Apr

Table S2 Statistical analysis results of the Generalized linear mixed models adjusted to evaluate the abundance predators in Bt soybean and refuge areas

Group	Technology	Mean ± SE	Phenol. stage	Mean ± SE
Araneae	**Bt**	2.63 ± 1.83 A	**Vegetative**	3.37 ± 2.38 A
	Refuge	3.25 ± 2.26 A	**Reproductive**	2.53 ± 1.75 A
	F value	*2.16*	***F value***	*2.32*
	P value	*0.1442*	***P value***	*0.1305*
Coccinellidae	**Bt**	0.13 ± 0.14 A	**Vegetative**	0.12 ± 0.14 A
	Refuge	0.16 ± 0.18 A	**Reproductive**	0.18 ± 0.19 A
	F value	*0.28*	***F value***	*0.51*
	P value	*0.5971*	***P value***	*0.476*
Heteroptera	**Bt**	0.84 ± 0.67 A	**Vegetative**	0.96 ± 0.79 A
	Refuge	1.10 ± 0.87 A	**Reproductive**	0.96 ± 0.75 A
	F value	*1.1*	***F value***	*0*
	P value	*0.3031*	***P value***	*0.9987*
Chrysopidae	**Bt**	0.47 ± 0.14 A	**Vegetative**	0.37 ± 0.14 A
	Refuge	0.37 ± 0.11 A	**Reproductive**	0.46 ± 0.13 A
	F value	*1.15*	***F value***	*0.54*
	P value	*0.2854*	***P value***	*0.4648*

Table S3 Statistical analysis results of the Generalized linear mixed models adjusted to evaluate the abundance of herbivores in Bt soybean and refuge areas

Group	Technology	Mean ± SE	Phenol. stage	Mean ± SE	Inter. Tech x Pehnol*	Mean ± SE
Caterpillars	**Bt**	0.05 ± 0.04 A	**Vegetative**	0.11 ± 0.08 A		
	Refuge	1.17 ± 0.53 B	**Reproductive**	0.55 ± 0.24 B		
	F value	*29.28*	***F value***	*7.21*		
	P value	*<0.0001*	***P value***	*0.0076*		
Stink bugs	**Bt**	1.52 ± 0.31	**Vegetative**	1.05 ± 0.33	**Vegetative Bt**	.66 ± 0.34 A
	Refuge	1.17 ± 0.33	**Reproductive**	1.69 ± 0.25	**Reproductive Bt**	.07 ± 0.34 B
	F value	*0.67*	***F value***	*2.28*	**Vegetative Refuge**	.66 ± 0.56 A
	P value	*0.4154*	***P value***	*0.1323*	**Reproductive Refuge**	.39 ± 0.26 A
					F value	*4.26*
					P value	*0.0398*

Means within a column followed by different letters are significantly different ($P < 0.05$, Tukey-Kramer test)
**Means comparisons were made within technology*

Table S4 Statistical analysis results of the Generalized linear mixed models adjusted to evaluate the richness of predators and stink bugs in Bt soybean and refuge areas

Group	Technology	Mean ± SE	Phenol. stage	Mean ± SE
Predators	**Bt**	1.98 ± 0.82 A	**Vegetative**	2.17 ± 0.91 A
	Refuge	2.05 ± 0.85 A	**Reproductive**	1.87 ± 0.77 A
	F value	*0.23*	***F value***	*2.5*
	P value	*0.6328*	***P value***	*0.1145*
Stink bugs	**Bt**	0.52 ± 0.10 A	**Vegetative**	0.43 ± 0.10 A
	Refuge	0.55 ± 0.10 A	**Reproductive**	0.67 ± 0.11 B
	F value	*0.23*	***F value***	*4.32*
	P value	0.6335	***P value***	*0.0384*

Means within a column followed by different letters are signifcantly diferent ($P < 0.05$, Tukey-Kramer test)

7.2 ANEXOS CORRESPONDIENTES AL ARTÍCULO 3

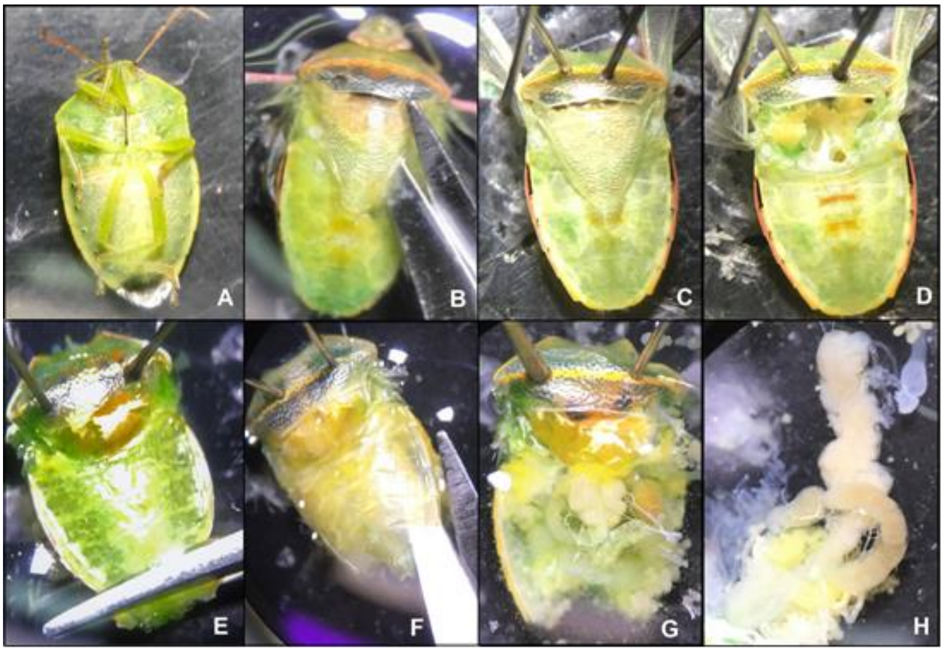

Figure A1. *Piezodorus guildinii* adult dissection procedure.

Figure A2. ELISA detection test (Quickstix, EnviroLogix®, Portland, ME) used to identify the presence of the Cry1Ac transgenic protein in the digestive tract, body, feces and eggs of six stink bugs adults. The first three showed a negative result and the next three a positive one.