Increasing the feasibility of General Surveillance of Genetically Modified maize

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TESI DOCTORAL

Increasing the feasibility of General Surveillance of Genetically Modified maize

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To my family,

Specially my mother

One planet, one experiment.

― Edward O. wilson

“And Man created the plastic bag and the tin and aluminum can and the cellophane wrapper and the paper plate, and this was good because Man could then take his automobile and buy all his food in one place and He could save that which was good to eat in the refrigerator and throw away that which had no further use. And soon the earth was covered with plastic bags and aluminum cans and paper plates and disposable bottles and there was nowhere to sit down or walk, and Man shook his head and cried: "Look at this Godawful mess.”

― Art Buchwald

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Resum

Els cultius Modificats Genèticament (MG) podrien tenir efectes negatius sobre el medi ambient, és per això que a la Unió Europea és obligatori el seguiment post-comercialització a llarg plaç, anomenat Seguiment General (SG), “General Surveillance-GS” en anglès. Malgrat aquesta obligatorietat, el SG que s’està duent a terme actualment no té la capacitat necessària per a detectar els efectes ambientals dels cultius MG. Per això, l’objectiu de la tesi és millorar la capacitat de detecció del SG a través de: a) la selecció d’artròpodes no-diana adients per al seguiment del panís MG, i b) identificar les xarxes de seguiment ambiental existents en el territori i determinar si les dades que generen tindrien la sensibilitat necessària per a detectar efectes ambientals del maneig agrícola a llarg plaç, com per exemple del cultiu del panís MG.

Dos dels tàxons més adients per al seguiment dels possibles efectes ambientals del panís MG són les papallones (Lepidoptera:Papilionoidea) i els caràbids (Coleoptera:Carabidae), ja que podrien ser molt sensibles tant als impactes dels panissos resistents a insectes (*Bt*) com als tolerants a herbicides (HT).

Per a determinar l’abundància, la variabilitat i la distribució de papallones i caràbids en l’agroecosistema del panís, durant dos anys es van mostrejar diferents hàbitats i localitats de tres zones de cultiu de panís al nordest de la Península Ibérica.

Les papallones es van mostrejar mitjançant recomptes visuals i van ser abundants en tots els hàbitats mostrejats. La majoria de les espècies observades estarien exposades als efectes del cultiu del panís MG perquè les plantes larvàries eren presents en tots els hàbitats, incloent els camps de panís. Es va desenvolupar i aplicar un sistema per a seleccionar les papallones més adients per al seguiment dels impactes del panís MG. Les espècies més adients depenien de la regió geogràfica, però els indicadors que es podrien mostrejar amb menys esforç en les tres regions serien les agrupacions d’espècies i les espècies *Polyommatus icarus* (Rottemburg) i *Pieris napi* (L.).

Els caràbids es van mostrejar mitjançant trampes de gravetat i van ser més abundants en els marges dels camps de panís. L’espècie indicadora més adient va ser l’omnívor *Pseudoophonus rufipes* (De Geer), ja que va satisfer els criteris d’abundància, rellevància, sensibilitat i facilitat de mostreig. Seria recomanable realitzar el seguiment del grup de caràbids carnívors com a indicadors de biodiversitat i control biològic d’invertebrats.

La xarxa de seguiment ambiental més adient va ser la xarxa de seguiment de les papallones de Catalunya (CBMS). Es van analitzar dades obtingudes de 29 punts de mostratge durant un período de 15 anys. Aleshores es va determinar la capacitat de la prova-*t* de Welch per a detectar diferències en l’abundància de 12 indicadores en dos tipus de paisatge. La capacitat de detecció va ser molt bona per a alguns indicadors (es podria detectar un canvi poblacional del 30%). La capacitat de detecció depenía del tamany mostral i de la variabilitat i augmentava ràpidament quan s’agregàven espècies i anys de dades.

La conclusió general d’aquesta tesi és que el SG dels panissos MG es podria millorar considerablement a través del mostratge de papallones i caràbids. A més, les dades generades per les xarxes de seguiment de papallones permetrien augmentar força la capacitat de detecció d’efectes ambientals.

Summary

Genetically Modified (GM) crops could adversely affect the environment, therefore long-term post-market monitoring (General Surveillance, GS) is mandatory in the EU. However, in its current form, GS lacks the required sensitivity to detect environmental effects of GMs. The aim of this thesis is to increase the effect detection capacity of GS through a) selection of suitable non-target arthropods (NTAs) to monitor GM maize, and b) identify suitable environmental surveillance networks (ESNs) and determine if their data is sufficiently sensitive to detect long term environmental effects of agricultural practices –such as GM cultivation.

Butterflies (Lepidoptera: Papilionoidea) and carabids (Coleoptera:Carabidae) were identified as the most appropriate surrogate taxa for monitoring effects of GM maize on non-target organisms due to their potential sensitivity to impacts of GM maize expressing insect resistance (*Bt*) or herbicide tolerance (HT). A two-year field survey was carried out in different maize agroecosystems in the north-east of the Iberian Peninsula to determine abundance, variability and distribution of carabids and butterflies across different habitats and sites.

Butterfly adults were sampled by transect-counts and they were abundant in all habitats sampled. Most species could be exposed to effects of GM maize because their larval host plants were present in maize fields and neighbouring habitats. A step-by-step selection procedure was developed and applied, finding that the most appropriate species for monitoring GM effects depended on the region considered. Of these, the indicators requiring the lowest sampling effort were the multispecies pools and the single species *Polyommatus icarus* (Rottemburg) and *Pieris napi* (L.).

Carabid adults were sampled by pitfall trapping and they most abundant in field margins. The best indicator was the omnivore *Pseudoophonus rufipes* (De Geer), satisfying criteria of abundance, relevance, sensitivity and ease of sampling. In addition, the carnivore group was a good indicator of biodiversity and invertebrate biological control.

The most suitable environmental surveillance network (ESN) in the study region was the Catalan Butterfly Monitoring Scheme (CBMS). A 15-year dataset from 29 recording sites was analysed to determine the capacity of Welch's *t*-test to detect differences in abundance of 12 farmland butterfly indicators across land-use types. Detection capacity was very good (a change below 30% could be detected) for some indicators and it depended mainly on sample size and variability of the data. Detection capacity rapidly improved when species were aggregated into multispecies indicators and when the longer time periods were used.

The main conclusion of this thesis is that GS of GM maizes could be substantially improved through field sampling of butterflies and carabids; and that data from butterfly monitoring schemes would allow to greatly increase effect detection capacity.

Resumen

El cultivo de plantas Modificadas Genéticamente (MG) podría tener efectos negativos sobre el medio ambiente. Por esta razón, en la Unión Europea es obligatorio el seguimiento post-comercialización de estos cultivos, llamado Seguimiento General (SG) o “General Surveillance-GS” en inglés. Sin embargo, en su forma actual, el GS carece de la capacidad necesaria para la detección de efectos ambientales de los cultivos MG. Así, el objetivo de esta tesis es potenciar la capacidad de detección del SG mediante: a) la selección de artrópodos no-diana que sean buenos indicadores para el seguimiento del maíz MG, y b) el análisis de datos generados por redes de seguimiento ambiental para determinar si tendrían la sensibilidad necessaria para detectar efectos ambientales derivados de prácticas agronómicas o de los cultivos MG.

La literatura indica que dos de los taxones más adecuados para el seguimiento de los posibles efectos ambientales del maíz MG son las mariposas (Lepidoptera: Papilionoidea) y los carábidos (Coleoptera: Carabidae), ya que pueden ser sensibles a los impactos de maíces resistentes a insectos (*Bt*) y tolerantes a herbicidas (HT). Durante dos años se muestrearon diferentes hábitats y localidades en tres zonas de cultivo maicero en el nordeste de la Península Ibérica para determinar la abundancia, variabilidad y distribución de las mariposas y carábidos en el agroecosistema del maíz.

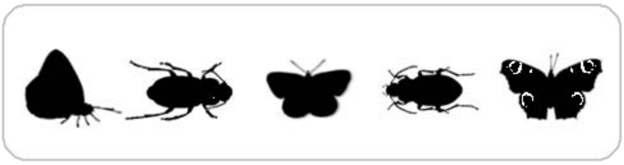
Las mariposas se muestrearon mediante recuentos visuales y fueron abundantes en todos los hábitats muestreados. La mayoría de las especies observadas podrían estar expuestas a los efectos del cultivo del maíz MG ya que se encontraron plantas nutricias de las larvas en todos los hábitats, incluidos los campos de maíz. A partir de los resultados de campo y de la literatura, se desarrolló y aplicó un sistema para seleccionar las mariposas más adecuadas para el seguimiento de los impactos del maíz MG. Las mejores indicadoras dependieron fuertemente de la región considerada, sin embargo, en este estudio las indicadoras que se podrían muestrear con el menor esfuerzo fueron los indicadores multiespecie, *Polyommatus icarus* (Rottemburg) y *Pieris napi* (L.).

Los carábidos se muestrearon mediante trampas de gravedad y fueron más abundantes en los márgenes de los campos de maíz por lo que éste sería la mejor localización para el seguimiento. El mejor indicador fue el omnívoro *Pseudoophonus rufipes* (De Geer), cumpliendo los criterios de abundancia, relevancia, sensibilidad y facilidad de muestreo. También se recomienda el seguimiento del grupo de carábidos carnívoros como indicador de biodiversidad y de control biológico de invertebrados.

La red de seguimiento ambiental que podría utilizarse con mayor facilidad es la red de seguimiento de mariposas de Cataluña (CBMS). Se analizaron los datos obtenidos en 29 puntos de muestreo durante un período de 15 años. Se determinó la capacidad de la prueba-*t* de Welch para detectar diferencias en la abundancia de 12 indicadores en dos tipos de paisaje. La capacidad de detección fue muy buena para algunos indicadores (pudiéndose detectar un cambio poblacional del 30%). La capacidad de detección dependía del tamaño muestral y de la variabilidad, y crecía rápidamente a medida que se agrupaban especies y años de datos.

La conclusión principal de la tesis es que el SG de los maíces MG se podría mejorar considerablemente a través del muestreo de determinadas mariposas y carábidos. Asimismo, se podrían utilizar los datos generados por las redes de seguimiento de mariposas ya que permitirían incrementar la capacidad de detección de efectos ambientales sin incurrir en elevados costes.

General Introduction



General Introduction

The first Genetically Modified (GM) crops reached the market in 1992. Currently, they are cropped on over 191.7 million hectares across the world and more than 18 million farmers a-year plant seeds containing this technology (Brookes & Barfoot, 2018). The main application of GM crops is for the management of weeds and pests of agricultural crops but there are an increasing number of other commercial traits present singly or stacked, ranging from abiotic stress tolerance, altered growth/yield and modified product quality, disease resistance and pollination control (ISAAA, 2020).

Since the very first GMO crops were produced, there has been concern regarding their safety for humans, animals and the environment at large. In this regard, one of the most controversial areas is the deployment of GM crops on a commercial scale, due to the potential and uncontrolled effects on the environment. Despite the extensive cultivation of GM crops and the numerous studies conducted on the environmental impacts of GM crops, this issue remains controversial (see reviews by Comas *et al.*, 2014; Kolseth *et al.*, 2015; Tsatsakis *et al.*, 2017; Yang *et al.*, 2017; Pellegrino *et al.*, 2018; Romeis *et al.*, 2019; Mandal *et al.*, 2020). Environmental risks of GM crops can be grouped into three main areas including risks associated with biodiversity; risks associated with gene flow and genetic recombination; and risks associated with development of resistance of target organisms (Tsatsakis *et al.*, 2017).

To safeguard human and animal health and the environment, before any GM crop is granted authorization to be released into the environment, they must overcome a long safety assessment process to prove their safety to human and animal health and to the environment. As part of the safety assessment process, before their commercial release, GM crops must undergo an environmental risk assessment (ERA) to ensure that they do not cause unacceptable detrimental effects to the environment. Although GM crops must overcome similar processes there are differences in the legislations and requirements for cultivation across the globe (e.g. AU, 2001; EC, 2001; USDA, 2004).

The Environmental Risk Assessment of GMOs in the EU

In the European Union (EU), has a specific legislation applicable to the commercialization and cultivation of GM crops (for updates to the legislation see https://ec.europa.eu/food/plant/gmo/legislation_en). The European legislation follows the "precautionary principle", as recommended by the Cartagena protocol, and is considered one of the most stringent regulations of GMOs in the world (Smyth, 2017), although the ERA procedures have also been criticized for being too lax (e.g. Dolezel *et al.*, 2018; Chvátalová, 2019).

At the EU community level, the competent authority in charge of safety assessment of GM crops is the European Food Safety Authority (EFSA) (EC, 2003). EFSA also has the obligation to publish guidance on the authorization process (for the relevant guidance and opinions emitted see https://www.efsa.europa.eu/en/topics/topic/gmo). The requirements that must be satisfied for authorizations to release GM organisms (GMOs) into the environment were set out in EU Directives 2001/18/EC (EC, 2001), recently updated by Directive (EU) 2018/350 (EC (European Comission), 2018). In addition, the use of GM crops for food and feed is set out in Regulation (EC) 1829/2003, and Regulation (EC) 1830/2003 concerning the traceability and labelling of GMOs. These main pieces of legislation are supplemented by implementing rules or by recommendations and guidelines on more specific aspects, emitted by EFSA. In addition, each member state in the EU has its own set of laws for the application of the EU Directives. For instance, in Spain activities with GMOs are regulated by "Ley 9/2003, de 25 de abril" and implemented through the "Real Decreto 178/2004, de 30 de enero", modified by chapter "Real Decreto 367/2010, de 26 de marzo" and "Real Decreto 191/2013, de 15 de marzo". These regulations set the legal context for confined use, deliberate liberation and commercialization of GMOs (https://www.mapa.gob.es/es/agricultura/temas/biotecnologia/omg/).

Companies seeking market approval of any GM crop must undergo an individual case-by-case science-based risk assessment (RA) to prove it is as safe as a non-GM counterpart; taking into account the direct, indirect, immediate and delayed effects, as well as the cumulative long-term effects, on human and animal health and the environment (EFSA, 2010).

One main area of concern addressed in the environmental risk assessment (ERA) is the potential adverse impact on non-target arthropods (NTAs) and the ecosystem services they provide, including biological pest control, pollination and nutrient recycling among others. The risk assessment of NTAs follows a tiered approach that focuses on the formulation and testing of clearly stated risk hypotheses, making maximum use of available data and using formal decision guidelines to progress between testing stages (or tiers) (Romeis *et al.*, 2008; EFSA, 2010). If the GM crop is considered to be as safe as its non-GM comparator, then authorization for cultivation and commercialization are granted, this license is valid in the entire EU for 10 years. Nevertheless, public opinion opposes deployment of GM in many EU countries (Bøhn *et al.*, 2012) and therefore in 2015 new regulations were issued to allow member states to opt out of GM crop cultivation (EC, 2015). As a result, GM crops are currently only cultivated in Spain and Portugal (ISAAA, 2019).

Post market environmental monitoring of GM crops

Even when environmental risk assessment is well planned and executed, it is impossible to know how a new GM crop will impact the receiving environment because of the complexity of the myriad interactions taking place in the receiving agrienvironments. For this reason, in addition to the pre-release environmental risk assessment, the ERA must include detailed plans for monitoring the crop once it is being cultivated in order to determine if there are any negative unintended environmental effects, this is known as post market environmental monitoring (henceforth PMEM).

The PMEM describes how the GM crop will be monitored to detect unintended adverse effects on the environment, the specific guidelines on how PMEM must be carried out are set out in the EFSA Guidance document (EFSA, 2011), supplemented with guidance on the use of statistical methods (Henrys *et al.*, 2014) and guidance on the use of data from existing environmental surveillance networks (ESN)(EFSA, 2014). The quality of the PMEM plans must be assessed by EFSA as part of the ERA and following authorization the PMEM must be implemented by the consent holder, the results are then evaluated on an annual basis by EFSA to assess the ongoing environmental safety of the GM crop.

The guidance on PMEM of GM plants (EFSA, 2011), describes two types of PMEM: Case-Specific Monitoring (CSM) and General Surveillance (GS).

CSM is conducted on a case-by-case basis and focuses on adverse effects that had been anticipated in the ERA. If CSM is considered necessary, it is usually only implemented during the time period necessary to address the areas of concern identified in the ERA.

GS on the other hand is compulsory and should be conducted for as long as the GM crop is cultivated. When monitoring measures are used, the EC regulatory framework proposes a comparative approach to detect GMO effects (GM crops versus a non-GM comparator). In addition, reference or baseline data should be recorded either prior to the market release of the GMO or in parallel (EC (European Comission), 2002). As part of the PMEM, GS aims to detect cumulative, delayed or unexpected adverse effects of GM crops on human health or the environment (EFSA, 2011). However, GS is not hypothesis-driven because it focuses on unanticipated effects, although it should be designed to identify aspects of the environment that need to be protected from harm. Currently, GS is recommended to use three main approaches(EC, 2001; EFSA, 2011): (1) monitoring the crop and its cultivation sites through a farm questionnaire; (2) using data collected by relevant existing environmental surveillance networks (ESN) and (3) compiling and analyzing data published in scientific literature. These approaches are recommended to be integrated in a framework of general environmental protection monitoring (Wilhelm *et al.*, 2010; EFSA, 2014). Recognizing that the GS implemented lacked the capacity to detect potential adverse effects of GM crop cultivation, EFSA published further guidance on data quality, management and statistical analysis (Henrys *et al.*, 2014) and on how data from the existing environmental surveillance networks (ESN) could be used for GS (EFSA, 2014).

The cultivation of GM crops in the EU: *Bt* maize MON810

In the EU, the only GM crop cultivated commercially is *Bt* maize MON810 (mainly in Spain but also in Portugal) which expresses a truncated form of the Cry1Ab toxin from *Bacillus thuringiensis* subsp. *Kurstaki* conferring resistance to Lepidopteran stemborers *Ostrinia nubilalis* (Hübner) and *Sesamia nonagrioides* (Lefebvre). Maize is the most important cereal crop worldwide and it is the second most important GM crop in area cultivated. The most important GM traits in maize are Herbicide Tolerance (GMHT) and Insect Resistance (*Bt*), which can be present singly or stacked in different maize varieties together with other traits (ISAAA, 2019). In the EU maize is the second most important cereal crop, used mostly as animal feed. EU production (grain and corn cob mix) in 2017 amounted to 64.7 million tonnes (EUROSTAT, 2019) but it was still necessary to import around 15 million tons of grain, most of which was GM (ISAAA, 2019).

In Spain, the pressure of cornborers is high, for this reason *Bt* maizes conferring resistance to Lepidopteran cornborers have been consistently planted on a commercial scale since 1998 (Eizaguirre *et al.*, 2006). From 1998 until 2002 only Event 176, expressing Cry1Ab toxin (Syngenta Seeds) was cultivated but registration of this event expired in 2007 and was not renewed. Since 2004 cultivation of event MON810 became prevalent in Spain (Eizaguirre *et al.*, 2006) and in 2018 the surface planted to MON810 maize was 115,246 hectares (ISAAA, 2019). Deployment of MON810 is uneven across Spanish territories, being most important in the communities of Aragón (42,645.74 ha) and Catalunya (36,429.89 ha) (MAPA, 2019).

The General Surveillance plan for MON810

A PMEM plan for the *Bt* maize varieties derived from Event *Bt*176 was carried out during the period 1998-2005, and a second monitoring plan for *Bt* maize varieties derived from Event MON810 was initiated in 2003. Both of these considered case-specific monitoring (CSM) for the evolution of resistance in target insects (Ives & Andow, 2002; Eizaguirre *et al.*, 2004; Farinós *et al.*, 2018) as well as initially for the potential effects on non-target arthropods (Ortego *et al.*, 2009). However, currently PMEM contemplates CSM only for resistance development in the target pests and GS for assessment of the remaining unintended effects on the environment.

The GS of MON810 consists only of the annual assessment of farm questionnaires (FQ) and reviews of scientific literature, despite reiterated recommendations to incorporate data from ESNs (EC, 2001; EFSA, 2017). From the annual GS reports presented annually by Monsanto, EFSA has concluded that results did not indicate any unanticipated adverse effects on human and animal health or the environment, but this conclusion may lack scientific basis as only very large effects could be detected with the assessment endpoints and methods used in the GS for MON810.

The farmer questionnaires used in the GS plan are designed to collect information on four specific areas: (1) area cropped to maize; (2) typical agronomic practices; (3) observations of maize MON 810; and (4) implementation of maize MON 810 specific measures. The farm questionnaires are designed to assess agronomical impacts of

MON810; for instance they could allow to indirectly analyse biological control functions by surveying outbreaks of maize herbivores (Sanvido *et al.*, 2009). However, it is unlikely that farm questionnaires would allow to assess impacts on biodiversity *per se* or ecological functions that are not translated into noticeable agronomic performance of the crop because most farmers lack the necessary training.

The second pillar of GS is the compilation and review of relevant scientific literature following guidelines published by EFSA (EFSA European Food Safety Authority, 2019). Scientific research depends on private and public funding and may not focus on any relevant aspect related to the cultivation of MON810. Certainly, there is no reason for scientific literature to continuously contribute solid data on changes to the agroecosystems derived from cultivation of GM crops.

In the third place, EU regulations recommend consent-holders to make use of existing networks involved in environmental monitoring (EC (European Comission), 2001, 2002), further guidance was given by EFSA regarding the available networks and the feasibility of their use (EFSA, 2014) and the statistical methods that could be used (Hails *et al.*, 2012; Henrys *et al.*, 2014). Despite this, so far the MON810 annual reports have not analysed existing monitoring networks in the EU because they considered them to be of less additional value than the other approaches (EFSA, 2017).

Clearly, the GS plan currently implemented for MON810 would be incapable of detecting most unintended effects on the environment and particularly on NTA and it therefore requires improvement. Ideally a GS plan should be capable of detecting any unintended effect resulting from GMP cultivation (EFSA, 2011; EFSA, 2012) but this results in high costs which none of the stakeholders are willing to accept. This problem was already recognized by EFSA (EFSA, 2011) which is why they recommended that GS be included in a network of general environmental protection monitoring. A compromise must be made to find the most meaningful assessment endpoints and the easiest, most cost-effective methods to assess possible effects, this view has been taken into account throughout this thesis.

There are three main aspects to take into consideration in order to improve the feasibility of General Surveillance of GM crops: a) the GS plan should be designed to encompass all potential traits of the GM crop; b) the GS plan should focus on appropriate assessment endpoints; c) appropriate methods should be used for effect detection, for instance the use of existing environmental networks.

Currently MON810 is the only GM crop cultivated in the EU, mainly in Spain. However, it is very likely that in future other *Bt* maize crops will be deployed, expressing B toxins targeting primary or secondary Lepidoptera or Coleoptera pests. In addition to insect resistance, the other most frequent trait expressed by commercial GM crops is herbicide tolerance, this trait is also often stacked with insect resistance traits. Therefore, this thesis focuses on the development of a GS plan for maize expressing different *Bt* and/or herbicide tolerance. In the following sections, the *Bt* and GMHT maize will be described in order to understand how the crop may impact the environment.

In order to select the most appropriate assessment endpoints, these should satisfy the following criteria: the assessment endpoints should be a valued conservation goal, they should be in close contact with the GM crop cultivation and be sensitive to changes derived from deployment of GM crops, finally, they should allow for statistical testing. Environmental monitoring is costly which is why it is often not carried out. Many experts have recommended using data from environmental surveillance networks. In order to make GS feasible, I will focus on identifying and assessing the sensitivity of existing environmental surveillance networks (ESN).

Bt crops and impacts on the environment

Transgenic crops containing genes derived from *B. thuringiensis* reduce key crop pests and insecticide usage, promote biocontrol services, and economically benefit growers although in some cases their deployment has resulted in the development of *Bt*-resistance and increase of secondary pests (Tian *et al.*, 2012; Kranthi & Stone, 2020). Furthermore, as they produce insecticidal substances they have a great potential to damage non-target organisms, particularly those taxonomically related to the target organisms (e.g. Lang & Otto, 2010). However, in the field few effects of *Bt* crops have been found on NTA and when they are observed they tend to be of a low magnitude (Marvier *et al.*, 2007; Wolfenbarger *et al.*, 2008; Gatehouse *et al.*, 2011; Dang *et al.*, 2017; Tsatsakis *et al.*, 2017).

Bacillus thuringiensis, biocontrol of insect pests

The bacteria *Bacillus thuringiensis* (*Bt*) (Bacillales: Bacillaceae) are cosmopolitan gram-positive sporulating bacteria that can facultatively infect insect larvae and other organisms. Formulations based on *Bt* are used to control insect pests in agriculture and forestry, animal farming or for human health, accounting for approximately 75% of the global bio insecticide market (Sanchis, 2011). Hundreds of different *B. thuringiensis* strains have been isolated expressing over 300 different insecticidal proteins targetting insect larvae mainly in the Orders of Lepidoptera, Coleoptera and Diptera, but strains have been found active against Hemiptera and Hymenoptera and other invertebrates such as Gasteropoda, Nematodes, Protozoa and bacteria (Schnepf *et al.*, 1998; Kondo *et al.*, 2002; Wei *et al.*, 2003; Yudina *et al.*, 2007; Peña *et al.*, 2013; van Frankenhuyzen, 2013; Palma *et al.*, 2014). In laboratory studies, *Bt* toxins have also been found to have haemolytic activity (Naimov *et al.*, 2008) and specifically attack human cancer cells (Ohba *et al.*, 2009). In order to adapt to the hosts available, *B. thuringiensis* are known to produce at least four different types of insecticidal proteins: they secrete toxins in their vegetative phase (vegetative (Vip) and secreted (Sip)) and produce crystalline inclusion bodies in their sporulating phase (δ-endotoxins) containing Crystal (Cry) and cytolytic (Cyt) proteins (Palma *et al.*, 2014). The most widely known and applied for pest control are the Cry toxins, but even in this case the mode of action of these toxins is still not fully understood (Vachon *et al.*, 2012; Palma *et al.*, 2014; Albright *et al.*, 2016). In general lines, Cry toxins must be ingested by a susceptible host and solubilised in the its gut. Once solubilised, they are proteolytically activated by midgut proteases and the activated toxin binds to specific receptors on the epithelium cell membrane, destabilizing them and producing cell lysis. Extensive pore-formation in the insects' midgut membrane results in the death of the insect (Gill *et al.*, 1992; Soberón *et al.*, 2009; Pardo-López *et al.*, 2013). The insecticidal activity of *Bt* toxins is highly specific due to broad differences in insects' gut pH, midgut proteases and toxin receptors (Palma *et al.*, 2014) making them relatively safe to non-target organisms.

Bt crops and their impacts on non-target arthropods

Bt toxins expressed in transgenic plants could have increased negative effects on non-target arthropods (NTAs) compared to microbial *Bt* applications. Firstly, plant-produced toxins could be more toxic and have a broader activity spectrum. Secondly, exposure of

NTAs in the field could be much greater because the plants produce the toxins in all tissues throughout the season and toxins could have increased dispersal or persistence in the environment. Finally, the genetic modification could also result in unintended negative effects on NTAs, due to pleiotropic effects.

The success for insect pest control and the relative environmental safety of bacterial *Bt* toxins led scientists to introduce genes from *B. thuringiensis* into commercial crops to protect them from insect pests (Vaeck *et al.*, 1988). However, in order to achieve a high enough biocidal activity of *Bt* toxins expressed in the plant it was necessary to modify the microbial genes. Firstly, to increase the insecticidal activity of the proteins, the *B. thuringiensis* gene was truncated to express only the active form of the toxin, (Fischhoff *et al.*, 1987). Secondly, the DNA sequence of the microbial genes introduced had to be modified because of differences between the microbial and the plant mRNA processing and translation processes (Koziel *et al.*, 1993). Currently, in addition to truncated and modified versions of Cry proteins, the new plant breeding techniques have allowed to produce a broad array of novel chimeric genes derived from Cry and Vip toxins (e.g. Fang *et al.*, 2007).

One of the main concerns regarding the environmental effects of *Bt* crops is the fact that the activated form of the toxin expressed by the plants could have a wider activity spectrum on non-target organisms because some of the steps responsible for host specificity such as gut pH and specific proteolytic enzymes, are no longer required. In addition to this, some varieties express chimeric toxins that are not given in nature and stacked events express different combinations of the toxins that could interact.

Another concern regarding cultivation of *Bt* maize compared to use of microbial *Bt* is the increased exposure of non-target organisms due to the higher levels of *Bt* toxin and differential environmental fate (Zurbrügg & Nentwig, 2009). The Cry proteins can be expressed in all tissues of the plant although expression levels vary between plant tissues and even between different leaves on the same plant (Nguyen & Jehle, 2007; Székács *et al.*, 2010). The toxins can also be released into the rhizosphere through root exudates (Saxena *et al.*, 2004), and may also enter the environment through faeces of farm animals (Icoz & Stotzky, 2008). The dispersal of the toxin into the environment can take place through numerous mechanisms. Pollen can be dispersed by wind and deposited in or around maize fields (Messeguer *et al.*, 2006; Hofmann *et al.*, 2014) including natural habitats (Lang *et al.*, 2015) and the toxin expressed in the pollen can remain active for days (Ohlfest *et al.*, 2002). Organisms feeding on maize tissues may disperse and be consumed by predators and the toxins can move through the food webs throughout the environment. Another pathway through which *Bt* toxins can disperse into the environment is through seed dispersal or gene transfer either through pollination of related plants or transformation of bacteria.

Exposure to *Bt* toxins is greatest for species feeding on GM maize plants but non-target arthropods may also be exposed by ingestion of pollen (Stanley-Horn *et al.*, 2001; Peterson *et al.*, 2010) in or adjacent to maize fields (Pleasants *et al.*, 2001; Gathmann *et al.*, 2006a; Hofmann *et al.*, 2010; Lang & Otto, 2015); exposure to root exudates and plant remains in the soil (Icoz & Stotzky, 2008; Mandal *et al.*, 2020) or in the water (Swan *et al.*, 2009), consumption of contaminated prey (Obrist *et al.*, 2006a, 2006b) or lower numbers and quality of prey (Meissle *et al.*, 2005; Romeis *et al.*, 2014).

Effects of Bt maize on non-target Lepidoptera

Non-target Lepidoptera are highly relevant in the risk assessment of *Bt* maize producing Lepidopteran toxic proteins because they are taxonomically close to the target insects. There are many species of non-target Lepidoptera present in maize agroecosystems (Losey *et al.*, 2003; Gathmann *et al.*, 2006b; Van Wyk *et al.*, 2007), particularly in field margins (Lang *et al.*, 2011; Wallis de Vries *et al.*, 2017). Some species can feed on maize, this is mainly the case of secondary pests, but the main route of exposure for most species is through ingestion of pollen deposited on their host plants. There is a large body of evidence that ingestion of Lepidopteran resistant *Bt* maize pollen is harmful to non-target Lepidoptera (see reviews by Duan *et al.*, 2010; Lang & Otto, 2010). Adverse effects depend on the amount of *Bt* toxin ingested and the larval susceptibility which can vary between species (e.g. Hellmich *et al.*, 2001a; Zangerl *et al.*, 2001; Lang & Vojtech, 2006; Felke *et al.*, 2010; Schuppener *et al.*, 2012). There is very little knowledge on the eventual ecological significance of the adverse effects in the field; for the well-known case of the Monarch butterfly *Danaus plexippus* (L.) they were considered of little significance (Gatehouse *et al.*, 2002) but they could be much more important for other species (Holst *et al.*, 2013). Recognising the potential risks of *Bt* maize pollen to NT Lepidoptera in protected habitats (Lang *et al.*, 2015), EFSA issued recommendations to reduce proximity of maize fields to 20-30 m (EFSA, 2015). In addition, mathematical models have been constructed to inform the ERA of *Bt* maize (Perry *et al.*, 2010, 2012) and to explore how pollen-mediated effects could impact butterfly populations in the field (Holst *et al.*, 2013; Fahse *et al.*, 2018).

Effects of Bt maize on non-target Coleoptera

Coleoptera are highly relevant in the risk assessment of *Bt* maize producing Cry3 proteins toxic to Corn Rootworm, (Chrysomelidae: *Diabrotica* sp.) because they are taxonomically close to the target insects. Non-target Chrysomelidae are rarely important in maize agroecosystems so *Bt* effect assessment has focused on other Coleoptera that are more abundant and important to biological pest control, such as ladybirds (Coccinellidae), ground beetles (Carabidae) and rove beetles (Staphylinidae) (Rauschen *et al.*, 2010); or to nutrient recycling, such as dung beetles (Scarabeidae).

Coccinellidae can take up the toxin directly from pollen or indirectly through prey (Obrist *et al.*, 2006a; Harwood *et al.*, 2007). When ladybirds were directly exposed to *Bt* toxins in laboratory studies, there were only adverse effects in the case of *Adalia bipunctata* (L.), whose larvae showed a significantly higher mortality rate when reared on food containing microbial Cry1Ab compared to the controls (Schmidt *et al.*, 2004), the same was observed in a later study when *A. bipunctata* fed on activated microbial Cry1Ab and Cry3Bb compared to controls (Schmidt *et al.*, 2009). Other species tested showed no adverse effects when fed *Bt* proteins through diet or pollen. *Stethorus punctillum* (Weise) was not affected when fed Cry1Ab or Cry3Bb proteins or Cry1Ab-expressing maize (Schmidt *et al.*, 2004); and there were no effects of feeding ladybirds on *Bt* maize pollen compared to non-*Bt* maize pollen: *Henosepilachna vigintioctopunctata* (Fabricius) on Cry1Ab *Bt* maize pollen (Shirai, 2006); *Propylea japonica* (Thunberg) on pollen containing Cry1e (Li *et al.*, 2017), pollen containing Cry1Ac/Cry1Ab (Xie *et al.*, 2019), or protein Vip3Aa (Zhao *et al.*, 2020); or pollen expressing Cry3Bb1 on *Coleomegilla maculata* DeGeer (Duan *et al.*, 2002; Ahmad *et al.*, 2006). There were no prey-mediated effects when ladybirds were fed contaminated prey. *S. punctillum* was not affected when it preyed on *Tetranychus urticae* Koch fed on Cry1Ab-expressing maize (Álvarez-Alfageme *et al.*, 2009). *P. japonica* was not affected when preying on aphid *Rhopalosyphum maidis* (Fitch) fed on maize expressing Cry1Ab (Yinghua 2019) and *Harmonia axyridis* Pallas was not affected in bi- and tritrophic feeding experiments using *T. urticae* and *R. padi* fed on a stacked *Bt* maize expressing Cry1A.105, Cry1F, Cry3Bb1,Cry34Ab1 and Cry2Ab2 (Svobodová *et al.*, 2017). In the field, no effects of *Bt* maize expressing Cry3Bb1 were observed on predators *C. maculata*, *Hippodamia convergens* Gurin-Meneville, and *Scymnus* spp (Ahmad *et al.*, 2006).

Carabids, also known as ground beetles (Carabidae) ingest Cry toxins in the field but uptake varies between species (Harwood *et al.*, 2006; Peterson *et al.*, 2009; Priesnitz *et al.*, 2013) probably due to their broad range of feeding habits, from carnivorous to phytophagous. No direct effects were observed when carabids were fed *Bt* pollen, Cry3Bb1 maize pollen had no effects on *Harpalus pensylvanicus* DeGeer or *Harpalus caliginosus* (Fabricius) (Ahmad *et al.*, 2006). No prey-mediated effects were observed when *Poecilus cupreus* (L.) were fed on larvae of *Spodoptera littoralis* Boisduval reared on *Bt* maize expressing Cry1Ab compared to non-*Bt* maize (Álvarez-Alfageme *et al.*, 2009). Finally, carabids are frequently sampled through pitfall traps for effect assessment of *Bt* maize but no effects have been observed in the field for a broad range of *Bt* toxins (e.g. De La Poza *et al.*, 2005; Priestley & Brownbridge, 2009b; Albajes *et al.*, 2012a; Priesnitz *et al.*, 2013; Skoková Habuštova *et al.*, 2015; Twardowski *et al.*, 2017)

Rove beetles (Staphylinidae) are mainly predaceous coleopterans that are frequent in agroecosystems. In laboratory experiments, there were no effects on *Dalota coriaria* Kraatz using the red spider mite *T. urticae* as prey in tritrophic bioassays and no enzymatic response was observed (García *et al.*, 2010). Moroever, field experiments assessing the effects of *Bt* maize on Staphylinidae found no effects of Cry1Ab maize (Twardowski 2014) or *Bt* maize expressing Cry3Bb1 (Svobodová *et al.*, 2016).

Differential effects of *Bt* crops in the field have been found for dung beetles (Scarabeidae) in the composition of the dung beetle community (Campos 2015) and differences in morphometric parametres of the dung beetle C*anthon quinquemaculatus* Castelnau were found in *Bt* maize compared to neighbouring natural habitats, that did not occurr in conventional maize (Alves & Medina Hernández, 2017). Finally, C*anthon rutilans* Castelnau and *Coprophaeneus saphirinus* Sturm fed on pig faeces from MON810 were less efficient in dung removal and burial (Campos *et al.*, 2018).

Chrysomelids belong to the same family as the target species of Diabrotica resistant maize. In laboratory studies, the colorado potato beetle *Leptinotarsa decemlineata* Say was found to be sensitive (Meissle & Romeis, 2009a) but other Chrysomelids such as *Galerucella vittaticollis* Baly were not affected by *Bt* maize pollen expressing Cry1Ab at field concentrations (Shirai, 2006). In field studies, there was no effect of a stacked *Bt* maize producing Cry1Ab, Vip3Aa20 and mCry3A on abundance of the corn flea beetle, *Chaetocnema pulicaria* (Melsheimer) (Hernández-Juárez *et al.*, 2018).

Pollinators

No effects of *Bt* toxins have been found on honeybees (*Apis mellifera* L.) fed on maize pollen expressing Cry1Ab (Rose & Dively, 2007), a stacked variety expressing Cry1A.105 and Cry2Ab2 (Hendriksma *et al.*, 2011) or to pollen expressing Cry1Ac/Cry1Ab fusion gene (Xie *et al.*, 2019). No effects were found either for purified plant-produced toxins Cry1A.105 and Cry2Ab2 on honeybee larvae (Hendriksma *et al.*, 2012). Regarding other bee species, no effects of *Bt*-sweetcorn pollen expressing Cry1Ab (*Bt* 11) compared to non-*Bt* sweetcorn were found on *Bombus terrestris* L. (Malone *et al.*, 2007).

Herbivores

Different Aphid (Homoptera: Aphidae) species are common on maize but as they are phloem feeders they take up a very low concentration of *Bt* toxin (Ramirez-Romero *et al.*, 2008; Romeis & Meissle, 2011) although some species may take up more (Burgio *et al.*, 2007). No effects of *Bt* maize expressing Cry1Ab compared to non-*Bt* maize have been found on *Sitobion avenae* Fabricius (Ramirez-Romero *et al.*, 2008) or *Rhopalosiphum padi* (L.) and *R. maidi*s (Dutton *et al.*, 2002; Shu *et al.*, 2018), or of a stacked *Bt* maize variety expressing Cry1, Cry2, Cry3 and Cry34Ab1/Cry35Ab1 on *R. maidis* (Shu *et al.*, 2019). However, effects of *Bt* maize have been found in some cases. For instance, a higher abundance of *S. avenae* was found in commercial fields of Compa CB® (event 176 expressing Cry1Ab) compared to non-*Bt* fields during six years of field trials in the Ebro basin (Lumbierres *et al.*, 2004; Pons *et al.*, 2005; Eizaguirre *et al.*, 2006). In laboratory studies, Lumbierres *et al.* (2004) found that developmental and pre-reproductive times of the offspring of the first generation of alate *Rhopalosiphum padi* were shorter and the intrinsic rate of natural increase (rm) higher when aphids fed on *Bt* maize; they considered that this effect could be due to changes in host-plant quality due to pleiotropic effects of the genetic modification.

In contrast to aphids, spider mites (Acari:Tetrastichidae) can accumulate *Bt* toxin. *T. urticae* was found to contain four times more Cry1Ab toxin than the maize leaves they fed on (Obrist *et al.*, 2006a) although this higher concentration had no effects on their fitness (Dutton *et al.*, 2002). In another instance, carmine spider mites, *Tetranychus cinnabarinus* (Boisduval*)*, were found to decrease concentration of *Bt* toxins in leaves of maize plants expressing Cry3Bb1 and Cry1Ab (Prager *et al.*, 2014).

Auchenorrhyncha (Planthoppers and Leafhoppers) are common non-target organisms feeding on maize and can also serve as a source of Cry protein exposure to predatory arthropods. In the field, the leafhopper *Zyginidia scutellaris* (Herrich-Schäffer) (Homoptera:Cicadellidae) were more abundant on *Bt* maize expressing Cry1Ab compared to non-*Bt* maize in 6 years of field studies in the Ebro basin (Pons *et al.*, 2005; Eizaguirre *et al.*, 2006). However, in other studies no effects of *Bt* maize were observed on *Z. scutellaris* although this species represented 94% of all planthoppers on maize (Rauschen *et al.*, 2008).

Thrips (Thysanoptera: Thripidae) are very abundant in maize agroecosystems and feed on maize leaves and pollen but they are little studied. No effects were found in the only study I found in which *Bt* maize expressing Cry1Ab toxin had no effects on the thrips *Frankliniella tenuicornis* (Uzel) and toxin content in its body decreased 97% within the first 24 h (Obrist *et al.*, 2005).

Predators

Spiders (Araneae) play an important economic and ecological role as pest predators in various crops, including maize (Meissle & Lang, 2005). They are exposed to *Bt* toxins through contaminated prey or through consumption of plant and pollen. A laboratory bioassay showed no effects of Cry1Ab *Bt* maize pollen compared to non-*Bt* maize pollen on *Araneus diadematus* Clerck (Ludy & Lang, 2006a), no effects of *Bt* maize expressing Cry3Bb1 on the spider *Phylloneta impressa* (Koch) (Araneae: Theridiidae) (Meissle & Romeis, 2009b) or when the spider was fed on spider mites (*T. urticae*) and aphids (*R. padi*) reared on stacked *Bt* maize producing Cry1A.105, Cry1F, Cry3Bb1,Cry34Ab1 and Cry2Ab2 (Svobodová *et al.*, 2017). However, enzyme expression was altered in spiders *Ummeliata insecticeps* (Bösenberg & Strand) and *Pardosa pseudoannulata* (Bösenberg & Strand) fed on *Drosophila* reared on Cry1Ab (Zhou 2013). In a metanalysis of field studies, higher populations of foliar and epigeal spiders were found on *Bt* compared to non-*Bt* crops (Peterson *et al.*, 2011). However, in individual field studies, there were no effects on abundance of foliage dwelling spiders (Ludy & Lang, 2006b) or on abundance and diversity of epigeic spiders and harvestmen (Opiliones) (Řezáč *et al.*, 2010).

Predatory spider mites (Acari: Phytoseiidae) are important to controlling herbivorous mites but effects of *Bt* crops on these organisms have been little studied. In a study using *Bt* glyphosate-resistant maize stacked with Cry3Bb1 and Cry1Ab the predator mites *Phytoseiulus persimilis* (Athias-Henriot), avoided *T. cinnabarinus* that had fed on *Bt* maize (Prager *et al.*, 2014).

Neuroptera are common predators of insect pests in agricultural systems, *Chrysoperla carnea* Stephens has received a considerable amount of attention since Hilbeck *et al.*, (1999) found higher mortality of *C. carnea* larvae that preyed *O. nubilalis* and *S. littoralis* reared on a diet containing activated microbial Cry1Ab toxin and the protoxins of Cry1Ab and Cry2A compared controls. Dutton *et al.*, (2002) also found that mortality of *C. carnea* preying on *S. littoralis* reared on Cry1Ab maize was also higher than controls.

A later study showed there were no direct effects of feeding *C. carnea* on Cry1Ab toxin, suggesting that adverse effects are mediated by prey quality (Romeis *et al.*, 2004), similar results were found on *Bt* cotton (Lawo *et al.*, 2010). In tritrophic experiments with *Helicoverpa armigera* (Hübner) larvae reared on Cry1Ac, Cry1Ab, or Cry2Ab toxins, no binding of the toxins to midgut epithelium were observed (Rodrigo-Simón *et al.*, 2006). In other studies feeding *C. carnea* on aphids (*R. padi*) or spider mites (*T. urticae*) reared on *Bt* maize there were no effects of Cry1Ab (Lozzia *et al.*, 1998; Dutton *et al.*, 2002), or using a stacked *Bt* maize with Cry1A.105, Cry1F, Cry3Bb1,Cry34Ab1 and Cry2Ab2 (Svobodová *et al.*, 2017). On other *Chrysopa* species there were rarely effects and they were contrasting according to toxin and genetical modification event; *C. plorabunda* (Fitch) females fed on pollen from event 176 (Cry1Ab) lived more and females fed on pollen from MON810 (Cry1Ab) laid less eggs (Mason *et al.*, 2008). No effects were observed when feeding maize pollen expressing Cry1Ab/2Aj to *C. sinica* (Tjeder) nor of feeding maize pollen expressing Cry1Ac/Cry1Ab fusion gene to *C. nipponensis* (Okamoto) (Xie *et al.*, 2019).

Heteroptera are a mixed group regarding feeding preferences but many species are important to biological control of maize pests, particularly *Orius* sp. (Anthocoridae). In a laboratory study, *Orius majusculus* (Reuter) (Heteroptea: Anthocoridae) developed faster and had higher fecundity when fed on *Bt* maize Compa CB® (Event 176) Cry1Ab material compared to isogenic maize (Lumbierres *et al.*, 2012). However, no effects were found on *Orius insidiosus* (Say) fed on *Bt* maize expressing the Cry3Bb1 protein (Ahmad *et al.*, 2006) or for Cry3Bb1 protein (Duan *et al.*, 2008). And no effects have been found for other heteropterans such as mirids (Rauschen *et al.*, 2009).

Parasitoids

Bt crops have been found to have an important impact on the parasitoids of the target species, as can be expected due to the reduction of it's hosts numbers and fitness (Sisterson & Tabashnik, 2005). Moroever, interspecific interactions at higher trophic levels become increasingly complex. In some cases, adverse direct effects of Cry toxins were observed, but not in others. For instance no direct effects of feeding *Bt* maize pollen expressing Cry1Ab compared to non-*Bt* maize pollen were observed in *Trichogramma ostriniae* Pang & Chen, (Hymenoptera:Trichogrammatidae)(Wang *et al.*, 2007) but

Cotesia marginiventris (Cresson) (Hymenoptera: Braconidae) was adversely affected when fed Cry1Ab from the plant through an artificial diet (Ramirez-Romero *et al.*, 2007).

Host-mediated effects have been observed for many parasitoids, for instance *Campoletis sonorensis* (Cameron) (Hymenoptera: Ichneumonidae) reared on Cry1Ab *Bt* maize-fed *Spodoptera frugiperda* Walker were smaller than counterparts emerging from non-*Bt* fed larvae (Sanders *et al.*, 2007). Prey-mediated efects could depend on the prey species, for instance *C. marginiventris* was adversely affected when parasitising S. *littoralis* reared on *Bt* maize expressing Cry1ab (Vojtech *et al.*, 2005) but no effects were observed when parasitizing *S. frugiperda* (Ramirez-Romero *et al.*, 2007). Finally, no effects of maize expressing Cry1Ac were observed on *Macrocentrus cingulum* (Hymenoptera: Braconidae) parasitising the Asian corn borer, *Ostrinia furnacalis* (Guenée) (Wang *et al.*, 2017).

Host availability is another important aspect influencing parasitoid abundance in the field. For instance, higher populations of aphids (*R. padi*) on *Bt* maize expressing Cry1Ab compared to a non-*Bt* isoline that resulted in higher parasitism levels by *Aphidius colemani* (Dalman) (Hymenoptera: Braconidae) in the greenhouse (Górecka *et al.*, 2008); higher levels of aphid infestation in *Bt* maize compared to non-*Bt* maize provided *C. marginiventris* with honeydew thus enhancing parasitisation of *O. nubilalis* (Faria *et al.*, 2007). Conversely, for *T. ostriniae* parasitism rates were higher in non-*Bt* maize fields (Wang *et al.*, 2007) and parasitism of *O. nubilalis* by the tachinids *Lydella thompsoni* (Herting) and *Pseudoperichaeta nigrolineata* (Walker) were also higher in non *Bt* maize compared to Cry1Ab *Bt* maize (event 176) (Bourguet *et al.*, 2002). However, in some cases prey availability did not seem to be so important and recruitment of the corn borer specialist *Macrocentrus cingulum* Brischke (Hymenoptera: Braconidae) was greater on *Bt* maize compared to non-*Bt* maize (Pilcher *et al.*, 2006).

Detritivores

Most collembollans (Collembola) are soil-dwelling detritivores that contribute to nutrient recycling. Studies found no effects of *Bt* pollen expressing Cry1Ab/Cry2Aj on *Folsomia candida* Willem (Isotomidae) (Zhang *et al.*, 2017) or when feeding on *Bt* maize leaf material expressing Cry1Ab (Clark & Coats, 2006). No effects on *Protaphorura armata* (Tullberg) (Onychiuridae) following exposure to *Bt* maize litter expressing Cry1Ab and non-*Bt* maize leaf litter (Heckmann *et al.*, 2006). In the field no effects were observed on

Collembola biodiversity (Debeljak *et al.*, 2007) or on the decomposition of *Bt* maize leaf litters by soil arthropods including collembolla (Zwahlen *et al.*, 2003). Finally, there was one study that reported adverse effects on the aquatic larvae of the Chironomid fly *Chironomus dilutus* Shobanov (Diptera: Chironomidae) when exposed to root extract of Cry3Bb1 maize (Prihoda & Coats, 2008).

In addition to laboratory and field assays focusing on specific species or taxons, numerous field trials have been carried out to assess potential effects of *Bt* maize deployment compared to a non-*Bt* comparator on the non-target arthropod communities in maize. There were very rarely effects in the field and most trials showed no effects of *Bt* maize on natural enemies (Romeis *et al.*, 2009) or other non-target arthropods worldwide (Tsatsakis *et al.*, 2017) or in the study region of Southern Europe (e.g. Comas *et al.*, 2014; Arias-Martín *et al.*, 2018).

Herbicide Tolerant crops and impacts on the environment

The first GM herbicide-resistant (HT) crops were Roundup Ready crop lines containing a gene derived from *Agrobacterium* sp. strain CP4, encoding a glyphosate-tolerant enzyme, the so-called CP4 EPSP synthase (Funke *et al.*, 2006) engineered by Monsanto in 1996. The CP4 EPSP synthase results in glyphosate-tolerant crops, enabling more effective weed control by allowing post-emergent wide-spectrum herbicide application. Currently, the main GM crop cultivated worldwide continues to be glyphosate-tolerant maize (mainly event NK603, Monsanto's Roundup ready 2) (ISAAA, 2019). Nevertheless, although glyphosate-resistant crops continue to be prevalent, there are also maize varieties on the market tolerant to other herbicides such as glufosinate-ammonium, 2,4-D or Sulfonylurea (ISAAA, 2020) and other traits can be expected in the future.

Deployment of GMHT crops have provided increased weed control, higher economic benefits for farmers, and they have also resulted in the widespread adoption of no till or reduced till agriculture that reduce the carbon footprint and protect agricultural soils from erosion (Brookes & Barfoot, 2018). However, their deployment has resulted in the use of large amounts of chemicals, for instance glyphosate-based herbicides are the most heavily used herbicide in the world (Myers *et al.*, 2016). This has also led to field-evolved resistance to glyphosate in many weeds (Tsatsakis *et al.*, 2017). Concerns remain regarding potential adverse effects of the deployment of GMHT crops on human and animal health and the environment at large (Gill *et al.*, 2018; Ledoux *et al.*, 2020) and some authors have concluded that biodiversity in general is negatively affected by the cultivation of HR GM crops (Bohan *et al.*, 2005; Isenring, 2010; Lovei *et al.*, 2010). Nevertheless, the increased flexibility in weed management options could benefit arthropod biodiversity and ecological functions such as biological control if managed accordingly (Bigler & Albajes, 2011).

Effects of Herbicide Tolerant maize on arthropods

The adverse effects of GMHT maize on non-target arthropods may take place due to direct effects of the GM trait (although these have never been recorded) or the broad-spectrum herbicides but they are usually the consequence of indirect plant-mediated effects due to weed shifts caused by changes in herbicide application patterns (Lundgren *et al.*, 2009).

Direct effects of herbicides on arthropods

Glyphosate is a systemic broad-spectrum herbicide that enters the plant by foliar contact. Its mechanism of action involves disruption of aromatic amino acid biosynthesis by inhibition of the shikimate pathway (Amrhein *et al.*, 1980) in plants. The active ingredient glyphosate is a relatively inert substance that must be formulated with other ingredients that may also have adverse effects on organisms other than plants. For this reason, comparative studies with glyphosate, co-formulants and formulations involved are of increasing significance (Klátyik *et al.*, 2017a; Székács, 2017; Defarge *et al.*, 2018; Mesnage and Antoniou, 2018) and should be taken into account in the risk assessment (Székács & Darvas, 2018). Until recently Glyphosate was considered to be relatively harmless to animals (Franz *et al.*, 1997; Giesy *et al.*, 2000), but an increasing body of research is challenging this view (see for instance reviews by Gill *et al.*, (2018) and Székács & Darvas, (2018)).

Considering the great number of arthropods exposed to herbicides in the field, there is a considerably low body of knowledge on their possible effects on arthropods. In laboratory studies, deleterious effects of glyphosate were found on phytophagous spider mites (de Saraiva *et al.*, 2016), on the aphid *Metopolophium dirhodum* (Walker) (Saska *et al.*, 2016), some spiders (Benamú *et al.*, 2010; Evans *et al.*, 2010) but not on others (Michalková & Pekár, 2009), most hymenopteran parasitoids studied (Stecca *et al.*, 2016; Cruz *et al.*, 2017; Pontes *et al.*, 2020) with few exceptions (Carmo *et al.*, 2010; Pontes *et* *al.*, 2020)*;* on the Chrysomelid *Ceratoma arcuata* Olivier (Pereira *et al.*, 2018), on some Carabids (Michalková & Pekár, 2009; Evans *et al.*, 2010) but not on others (Brust, 1990) and on the Neuropteran predator *Chrysoperla externa* (Hagen) fed on treated Lepidoptera eggs (Schneider *et al.*, 2009). Recently glyphosate has been found to increase mortality of honeybees (Abraham *et al.*, 2018), negatively affect bee intestinal microbiota (Motta *et al.*, 2018; Vázquez *et al.*, 2018; Blot *et al.*, 2019) and impair cognitive and sensory capacities of the bees (Herbert *et al.*, 2014; Balbuena *et al.*, 2015; Farina *et al.*, 2019), despite this, bees do not appear to be repelled by sprayed flowers (Fagúndez *et al.*, 2016). However, glyphosate had no adverse effects on dragonfly larvae of *Aeshna cyanea* Müller (Ujszegi *et al.*, 2016).

Similarly, glufosinate-ammonium had adverse effects on some arthropods while it did not harm others. Glufosinate did not harm the red spider mite *T. urticae*, but it had adverse effects on its two predatory mites, as well as on predators *H. axyridis* and *Orius strigicollis* (Poppius) (Ahn *et al.*, 2009). The herbicide also increased the mortality of the Hesperid caterpillar *Calpodes ethlius* (Kutlesa & Caveney, 2001).

Indirect effects of GMHT maize deployment

The cultivation of GMHT crops can have strong effects on arthropods due to alterations in the abundance, composition and phenology of the adventitious plants present in the maize fields and their margins that insects use for food, shelter or other requirements (Norris & Kogan, 2005). Indirect effects on herbivores and arthropods from higher trophic levelss can occur when weed species interact with each other and with crop plants modifying plant physiology, chemical and visual cues and competing for resources, hence, weed management can interfere in many different ways with arthropods (Bigler & Albajes, 2011). Deployment of HT maize is expected to result in lower abundance and diversity of weeds and smaller populations of arthropods (Cerdeira & Duke, 2006) but this is not always the case, and in some cases weeds can be more abundant in HT maize compared to conventional maize. For instance Perry *et al.* (2004) found a larger weed abundance in glyphosate-resistant fodder maize than in conventional weed management. In this line, in a recent study comparing effects of different herbicide programs, Loureiro *et al.*, (2019) found no effects on the richness and species diversity of the weeds but they did fins differences in weed composition that could affect ecosystem services provided to the arthropods.

Effects of GMHT crop deployment are very complex as they can involve myriad interactions at many different levels, this makes it very difficult to establish causal links between differences in herbicide application regimes and arthropod populations in the field. One of the few cases when a clear causal link was established was regarding the reduction in monarch butterfly *D. plexippus* populations, clearly linked to the decrease in abundance of the larval host plant due to effects of increased herbicide applications across the US cornbelt (Pleasants & Oberhauser, 2012). In most cases, however, the effects of GMHT crops on arthropods are contrasting due to the complexity of interactions between entomofauna and plants. It is generally considered that a high diversity of weeds should lead to increased biodiversity of beneficial arthropods but this is not always the case. For example, higher density of predators has been reported at lower weed abundance, for instance in the case of general predators *Orius spp*. and spiders (Albajes *et al.*, 2009). Finally, most studies found contrasting results, for instance herbivores and parasitoids were more abundant on non-weedy plots, whereas cocinnellids were more abundant in weedy plots (Penagos *et al.*, 2003); herbivore pests were reduced in weedy polyculture plots (Gianoli *et al.*, 2006) but general on-plant predators between weedy and non-weedy plots was similar. In the largest field study ever carried out on GMHT crops, the UK Farm Scale Evaluations (Champion *et al.*, 2003), differences in arthropod abundances did not follow a clear trend and varied during the season and between seasons, crops and taxa (Hawes *et al.*, 2003; Roy *et al.*, 2003). In maize fields in NE Spain, studies also found that responsiveness of arthropods to differences in weed management associated to GMHT maize was taxon-specific (Aes *et al.*, 2009; Madeira & Pons, 2015) and varied according to the intensity of the weed changes (Albajes *et al.*, 2014).

Arthropod communities in maize: potential for monitoring effects of GM maize

When designing and implementing a monitoring plan aimed at detecting potential effects of GM maize cultivation on the environment, apart from selecting indicators suitable from an ecological and biological point of view, is is also essential to select indicators that are capable of detecting effects from a statistical point of view (Field *et al.*, 2007). In this regard, it should be possible to reliably measure differences of the indicator with/ without or before/after GM maize deployment. In order to achieve this, the statistical tests applied to the data must be capable of detecting the change if it occurs, in other words the data must yield adequate statistical power. The power of a test is the probability of rejecting the null hypothesis of no effects, when it is false and there are effects. The power measures the probability that the test will detect an effect of a known magnitude using a specified experimental design and it varies according to the magnitude of the effect specified (Perry *et al.* 2003). Inversely, the magnitude of an effect (the effect size) that a test is able to detect (detection capacity) may be calculated for a specified power. The effect size is usually obtained as the scaled difference between the density of the organism recorded on the GM variety and the density of the organism on a control non-GM variety called the comparator. A field test with a high detection capacity, for a given test power, is a test that is able to detect small significant differences between the GM variety and its non-GM control (Comas *et al.*, 2014).

Effect detection capacity of non-target arthropod taxa in the maize agroecosystems

Since the first commercial plantings of *Bt* maize in Spain, numerous field trials have been carried out in order to assess potential adverse effects on NTAs (Eizaguirre *et al.*, 2006; Ortego *et al.*, 2009; Comas *et al.*, 2014; Arias-Martín *et al.*, 2018). The sampling techniques most widely used were visual sampling of the epigeal fauna on maize plants, pitfall trapping for ground-dwelling arthropods and the use of yellow sticky traps to sample arthropods in the maize canopy. Data from these studies have been analysed in order to determine the statistical power or the detection capacity of the different arthropods and trapping techniques.

n field trials to assess potential impacts of *Bt* maize on predatory arthropods conducted in Spain, mainly in the Center and Northeastern Spain, Anthocoridae, Coccinellidae, and Araneae represented about 90% of the total number of predators recorded in visual samplings of maize plants whereas Carabidae and Araneae were the two prevalent predator groups collected in pitfall traps (de la Poza *et al.*, 2005; Farinós *et al.*, 2008) followed by Staphylinidae which were caught to a lesser extent (Farinós *et al.*, 2008).

The capacity to detect effects of a given taxon depends on the abundance, variability and number of replicates, in general high abundance and low variability would result in the greatest detection capacities and allow to reduce replication (Comas *et al.*, 2013). In addition, taxons with high abundance do not require such intensive sampling during the growing season (Comas *et al.*, 2015).

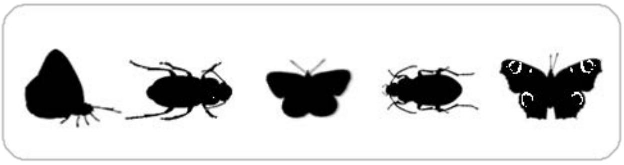
Based on the statistical power of the assays, the best NTAs for visual sampling were *Orius* spp. and Araneae and for pitfall trapping Carabidae, Araneae, and Staphylinidae . In yellow sticky traps, Arias-Martín *et al.*, (2018) found that the leafhopper *Z. scutellaris* and Mymaridae parasitoids (Hymenoptera) had the highest capacity to detect differences between *Bt* and non-*Bt* maize.

In a study to identify the most suitable NTAxa, considering trophic level, for detection of effects (below 50%) between GM maize and a non-GM comparator, the most suitable taxa included leafhoppers among herbivores; *Orius* spp., Araneae, and Carabidae among predators; chalcidids, particularly the family Mymaridae, among parasitoids; and Chloropidae as decomposers. The single most influential factor determining the relative detection capacity was taxon abundance (Albajes *et al.*, 2013).

Data from arthropod monitoring networks

Environmental monitoring plans are often too costly to implement because a high number of replications in space and time are needed for reliably detecting changes of the indicators (Field *et al.*, 2007). For this reason, in the EU there is considerable interest in using data collected by already existing survey networks (ESNs) for effect assessment of GM crops (Glandorf D. C. M., 2012; Hails *et al.*, 2012; Lang & Bühler, 2012; EFSA, 2014). However, few arthropods are monitored on a regular basis using a standardised monitoring protocol and there are relatively few ESN suitable for GS of GM crops (Smets *et al.*, 2014). Considering arthropods, the most promising ESN for effect assessment of GM crops are butterfly monitoring schemes, because they use a standardised monitoring protocol for collection of butterfly data and they are increasingly present throughout the EU (Schmucki *et al.*, 2016), including agricultural areas (Brereton *et al.*, 2010). Data from these butterfly monitoring schemes have been used to study effects of environmental change (e.g. Stefanescu *et al.*, 2009; Oliver *et al.*, 2014; Herrando *et al.*, 2016) and appear suitable for the assessment of GMO impacts (Lang & Bühler, 2012). Therefore, the use of ESN data for GS of GM crops is very promising but requires further study.

Objectives



Objectives

The General Surveillance of GM crops in Europe has many weaknesses that must be addressed, thus the aim of this thesis was to improve the feasibility of general surveillance of genetically modified maizes (namely GMHT and *Bt* maizes). In order to achieve this, I focused on two key aspects: a) identification of non-target organisms suitable for general surveillance of genetically modified *Bt* and GMHT maizes and b) identification and assessment of the sensitivity of regional environmental surveillance networks.

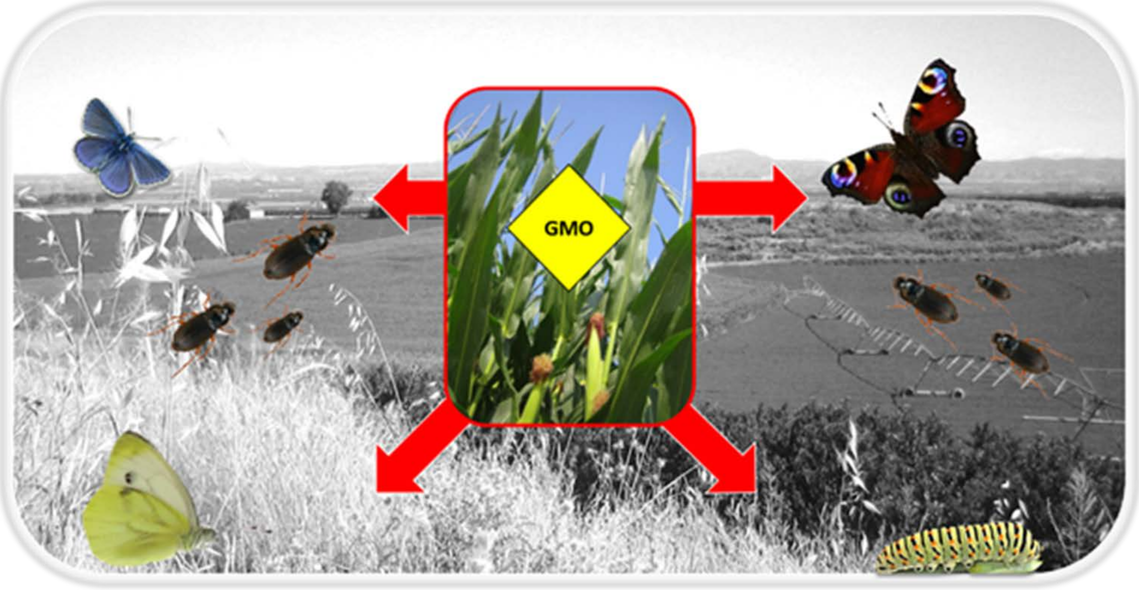


Fig. 1. Schematic representation of the objectives of the thesis.

After reviewing the state of the art of General Surveillance in Europe it was evident that it is necessary to monitor organisms in close contact with the GM crops. Taking this into consideration, the existing information on effects of GM crops on non-target arthropods was reviewed in order to identify those organisms most suitable for monitoring potential impacts of GM maizes. We found that Carabidae (ground beetles) and Papilionoidea (butterflies) were two groups with great potential because they carry out important functions in the maize agroecosystem, they are close to the target organisms and they respond to changes in the environment. In addition, other studies in Spain and elsewhere show that sampling these organisms could generate data with the high sensitivity required for effect detection in the field. Therefore, the first two objectives of the thesis were to determine which Carabidae and Papilionoidea could be used for field monitoring impacts of GM maizes in maize growing areas in the northeast of Spain.

Field monitoring has a high economic cost and therefore it is often not feasible to implement. It is for this reason that already existing environmental surveillance networks are often recommended monitoring schemes because the data they generate has a great potential for monitoring environmental change. In this regard, the third objective of the thesis was to identify the most suitable environmental surveillance networks and assess whether the data they generate has the required sensitivity for monitoring environmental effects of GM maizes.

Objective 1. Selection of appropriate butterflies for GS of GM maize

Butterflies (Papilionoidea and Hesperiidae) are excellent candidates for environmental monitoring because they are one of the best studied insect groups, they are good indicator organisms (Thomas, 2005), and a valued conservation goal (Van Swaay *et al.*, 1999). Most importantly, butterflies are key organisms for this purpose given their sensitivity to *Bt* toxins and their dependence on the flora that can be altered by GMHT maize cultivation. In Chapters 1 and 2, a feasible general surveillance plan is outlined for GM maize through the identification of suitable butterfly species or species pools for cost-effective and reliable monitoring of effects of *Bt* and GMHT maize on biodiversity in Mediterranean conditions. In order to do this, the following specific objectives were established:

a) Determine the distribution and abundance of common butterflies present in maize agroecosystems.

b) Determine the distribution and abundance of larval host plants in maize agroecosystems.

c) Identify larvae present at maize pollination and determine if sampling larvae could increase feasibility of butterfly monitoring for GS.

d) Develop and apply selection criteria to identify appropriate butterfly indicators for general surveillance of GM maize.

e) Determine the sampling effort involved in monitoring suitable butterfly indicators.

Objective 2. Selection of Carabids as indicators for GS of GM maize

Carabids are known to reflect environmental changes and they are common in maize agroecosystems in the study area where they carry out important ecological functions such as biological control of pests and eaters of weed seeds. Monitoring carabids could improve GM maize effect detection given their importance, their exposure to GM cropping and their sensitivity to environmental change. In Chapter 3, carabids are identified that could be monitored as part of GS to increase detectability of potential adverse effects of *Bt* and GMHT maize cultivation on ecosystem diversity and functions, the following specific objectives were established:

a) Determine the abundance and distribution of carabid species forming the carabid assemblages in the maize agroecosystem across habitats, regions and years.

b) Select appropriate carabid indicators for monitoring based on distribution, statistical power, sampling effort, and capacity for reflecting ecosystem diversity and functions.

c) Outline a feasible GS plan including carabids for monitoring GM maize.

Objective 3. Identify and assess sensitivity of ESNs for GS.

There are a number of already existing environmental surveillance networks (ESN) that collect environmental data. This data, with an appropriate statistical analysis, could be used to monitor effects of GM crops on the environment. In order to assess the practicability of this approach, the following specific objectives were established and discussed in Chapter 4:

a) Identify and analyze the Environmental Surveillance Networks that could be used for GS in the study area.

b) Identify the characteristics of the data that determine their effect detection capacity.

c) Assess the statistical sensitivity of the data generated by Environmental Surveillance Networks for detection of unintended effects of GM maize cultivation.

d) Identify suitable butterfly indicators for general surveillance of GM maize that could be monitored through Environmental Surveillance Networks.

Outline of chapters

Chapter 1: Butterflies for Post Market Environmental Monitoring of GM Maize in Spain

This first chapter reports the first results obtained regarding objectives 1 and 3. In the first place butterfly species that could be appropriate for general surveillance of maize were assessed from bibliography. I carried out a field study in three differentiated maize-growing regions in the NE of the Iberian Peninsula, using the transect-count method to determine the identity and distribution of butterfly species in different habitats typically associated to maize. Finally, the Catalan Butterfly Monitoring Scheme was identified as the most suitable environmental surveillance network for general surveillance of genetically modified maize.

Chapter 2: Sampling and selection of butterfly indicators for General Surveillance of Genetically Modified maize

In this chapter I focused on objective 1. I report the results from both years of the field study described in chapter 1. I also carried out a floral survey to determine the distribution of butterfly larval host plants in maize agroecosystems and report the results of a larval survey in maize field margins. I used the results from the field studies to develop a step-by-step selection process to identify the most suitable butterfly species for each of the regions studied and also assessed sampling effort required to reliably detect differences in butterfly indicator abundance.

Chapter 3: Monitoring carabid indicators could reveal environmental impacts of genetically modified maize.

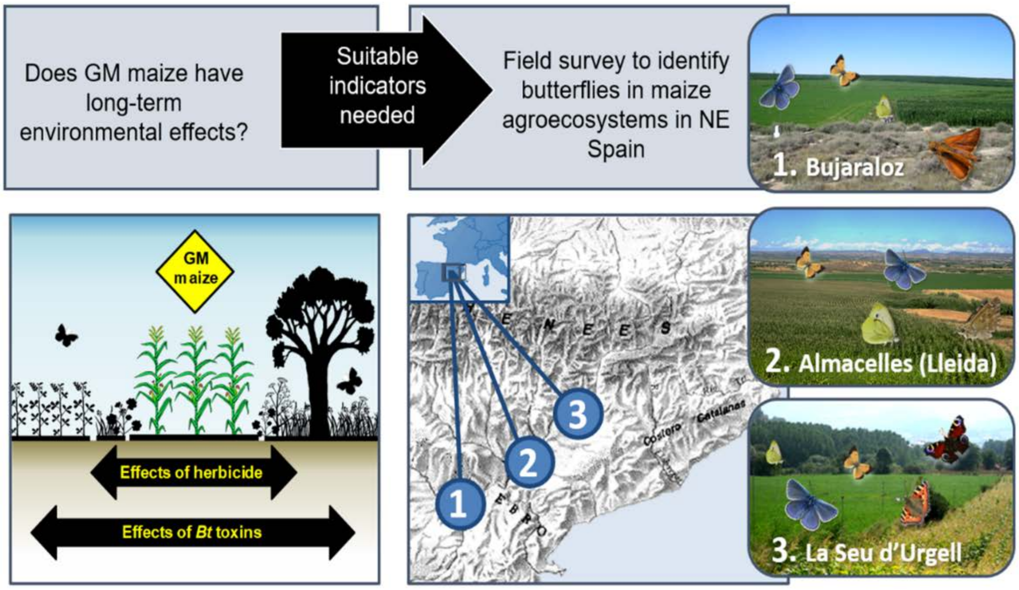
This chapter addressess objective 2. A two-year field study was carried out in three differentiated maize-growing regions order to determine the abundance and distribution of carabids in different habitats of the maize agroecosystem. Carabids were sampled by pitfall trapping and identified to species. I analysed the carabid communities and trophic groups across habitats, regions and years using community measures and variation partitioning. Finally, I used prospective power analysis to determine required sampling effort and took all results into account in order to propose the most appropriate carabid indicators for general surveillance of genetically modified maize.

Chapter 4. The Catalan butterfly monitoring scheme has the capacity to detect effects of modifying agricultural practices

Here I focus on objective 3, I analysed a large dataset generated by the Catalan Butterfly Monitoring Scheme in order to determine if it would be possible to reliably detect differences in butterfly abundance across different landscape types. I selected transects located in different landscape types using GIS. Then, I used Welch's *t*-test to compare the abundance of different farmland butterfly species and multispecies groups across the two landscape types. The difference in butterfly abundance that the *t*-test would be capable of detecting was assessed within the same year or across multiple years.

Chapter 1.

Butterflies for Post Market Environmental Monitoring of GM Maize in Spain



Butterflies for Post Market Environmental Monitoring of GM Maize in Spain

The only genetically modified (GM) maize planted in Europe is *Bt* maize resistant to Lepidopteran stemborers, cultivated on a large scale in Spain. Maize expressing herbicide tolerance and insect resistance traits singly or stacked will predictably also be cropped in the future.EC legislation demands that an Environmental Risk Assessment (ERA) for GM crops be carried out prior to release into the environment and that they be monitored after release by the implementation of Post Market Environmental Monitoring (PMEM) to detect possible adverse effects unanticipated in the ERA. Butterflies are often used for monitoring because they are sensitive to environmental changes, they are relatively easy to see and identify in most cases they are socially valuable. For PMEM of GM maize butterflies are particularly meaningful because they belong to the same taxonomic group as the target insect in the case of maize expressing *Bt* toxins and they feed on weeds that could be affected by the herbicide regime of HT maize. There is little information regarding butterfly species that can be exposed to the hazard of those two types of GM traits and which could be used for PMEM in southern European countries. This study addresses this knowledge gap and, after a bibliographic search and a year of field sampling, identifies some potential indicator species.

Other studies have found that the number of samples needed to detect even large changes in butterfly populations is impracticable. Thus, ways to integrate PMEM into existing monitoring schemes that cover large areas of Europe, such as the Butterfly Monitoring Scheme (BMS), are discussed.

Keywords: gmo, biotech crops, risk assessment, Lepidoptera.

Introduction

Maize is the second most important cereal crop in the EU, after wheat. In 2010 the EU produced 56.5 m tonnes of grain maize (EUROSTAT, 2010). In Spain, where GM maize has been grown since 1998, transgenic maize varieties have already reached 32 % of the total area cultivated (116,306 ha) in 2012 (MAGRAMA, 2012). Currently the only genetically modified (GM) maize cultivated in Europe is *Bt* maize carrying the event MON810.Authorisation of a considerable number of other events conferring insect resistance and herbicide tolerance to maize crops are in process. Consequently, in the future it is probable that different transgenic events will occur singly or stacked and that these GM crops will be present simultaneously in the same landscape.

Directive 2001/18/EC demands that an Environmental Risk Assessment (ERA) for GM crops be carried out prior to their release into the environment. The aim of the ERA is to identify direct, indirect, immediate or delayed potential adverse effects on human health or the environment. In addition, the notifier must present a Post Market Environmental Monitoring plan (PMEM) which must be into place once the crop is released into the environment (The European Parliament and the Council, 2001). There are two types of PMEM: Case-Specific Monitoring (CSM) and General Surveillance (GS). CSM focuses on anticipated adverse effects and is therefore only required if the ERA has identified potential risks. GS is always compulsory because it aims to detect unanticipated adverse effects and long-term cumulative effects that could not be detected in the ERA (EC, 2002). As its name implies, GS is not hypothesis driven, nevertheless, in order to make it practicable it is worthwhile to select some representative processes or organisms to be monitored.

The need for integrating and harmonising agents and institutions involved in surveillance has been recognised as an important issue (Wilhelm *et al.* 2009) but as yet is still under discussion. Currently, GS relies basically on farm questionnaires and therefore the information obtained on non-target organisms tends to be lacking or is very general. In addition, information from GS is not easily available, it is very segregated and most often results cannot be compared. For these reasons it is necessary to integrate GS in existing environmental surveillance networks, as already recognised by the European Food Safety Authority (EFSA).

Cumulative or unanticipated effects of GM maize on non-target organisms will depend, among other aspects, on the genetic modification. Currently the two types of GM maize that are liable to be cultivated in the EU are insect resistant and herbicide tolerant maize.

Bt maize has been modified by introducing genes from *Bacillus thuringiensis* that encode specific insecticidal Cry proteins. The only *Bt* maize cultivated at present is MON810 which is resistant to Lepidopteran pests such as the European corn borer (*Ostrinia nubilalis* (Hübner)) and the Mediterranean corn borer (*Sesamia nonagrioides* (Lefèbvre)).

Herbicide tolerant (HT) maize, on the other hand, has been engineered in order to confer resistance to broad spectrum herbicides among which glyphosate. HT maize implies the repeated use of non-selective herbicides throughout the cropping cycle in contrast to the single pre-emergence application which is current common practice.

Diurnal Lepidoptera, commonly called butterflies, are often used for environmental monitoring because they are sensitive to environmental changes in general due to their short generation time. They reflect changes in flora due to the larval herbivory. They are easy to monitor and are socially valuable. Finally, there are a number of butterfly monitoring schemes (BMS) throughout the EU and elsewhere making it possible to compare information on butterfly population dynamics and abundance.

For PMEM of GM maize, butterflies are particularly meaningful organisms. In the case of Lepidoptera-resistant maize they belong to the same taxonomic group as the target insect. Non-target species that are related taxonomically to the target pests are most likely to be affected by the protein. Thus selection of these taxa increases the likelihood of detecting a hazard if one exists (Romeis *et al.*, 2008). Negative effects on butterfly survival have been found for some *Bt* varieties (e.g. Dively *et al.*, 2004). The risk posed by Cry1Ab depends on the exposure of butterfly larvae to ingestion and their sensibility to the toxin. Maize is not a food plant to most non-target butterflies so the risk is due mainly to the accidental ingestion of the maize pollen by larvae. Despite the fact that the EFSA panel of experts asserted that the amounts of MON810 pollen grains in and around maize fields were unlikely to adversely affect a significant proportion of non-target Lepidoptera larvae, from which they concluded that no case-specific monitoring plan for non-target Lepidoptera was necessary (EFSA, 2012), cumulative or unanticipated effects may take place.

Lepidoptera also represent a good choice of indicator group for the monitoring of HT maize because larvae are herbivorous and depend directly on the presence and abundance of their food plants. The changes in herbicide regimes can alter the diversity and abundance of plant species depending on their sensitivity to the herbicides used (Heard *et al.*, 2006) and this may have deleterious effects on butterfly populations as reported for Monarch butterflies (Pleasants & Oberhauser, 2013). Besides, the spray bars will be higher for applications during later stages of the crop so it is probable that drift of herbicide will increase compared to conventional applications (Hilbeck *et al.*, 2008). Exposure of each species of butterfly will depend on the extent at which the food plant is controlled by glyphosate.

Although a number of studies have been and are being carried out to evaluate the risks of GM maize on non-target Lepidoptera (e.g. see the review on laboratory and field trials by Lang & Otto (2010)), there are few studies done under Mediterranean conditions. Additionally, a series of mathematical models have been developed in order to assess the risk of *Bt* maize expressing Cry1Ab (Perry *et al.*, 2010; Holst *et al.*, 2013) or Cry1F (Perry *et al.*, 2012) on non-target Lepidoptera. The drawback of these models is that they are not adapted to Spain because the indicator species that are used are the nettle feeding butterflies *Aglais urticae* (L.) and *Aglais io* (L.) and nettles (*Urtica* spp.) are not a common plant in most of the maize agroecosystems in Spain.

The objectives of this study are first to identify butterfly species which could be exposed to *Bt*-maize pollen in Spain and/or which could be affected by the change in herbicide regimes induced by HT maize. Secondly, this study aims to determine how to adapt existing environmental surveillance networks in order to improve both the data obtained and the long-term practicability of GS of GM maize in Spain.

Materials and methods

Identification of butterfly candidates from bibliography

There are a total of 169 species of Papilionoidea found in Catalonia and/or Aragón. Information was collected on the different species regarding their ecology and biology, the main source was (García-Barros *et al.*, in press). This information was then used to make a list of candidate species for each of the three study areas (Bujaraloz, Almacelles and La Seu) that could then be verified in the field. The candidates were selected according to risk criteria taking into account the exposition of larvae to possible effects of *Bt* and HT maize, conservation value and pest status (Schmitz *et al.*, 2003). Although susceptibility of each species to *Bt* may vary, this was not taken into account due to lack of information.

Butterfly species that could be exposed to the *Bt* toxin or affected by changes in herbicide regimes were selected according to the following:

- The species' geographical range should include at least one of the study areas.
- Butterfly habitats should occur close to maize ecosystems. According to (Messeguer *et al.*, 2006) most of the pollen falls at 20-25 m from the crop border. It is in this surrounding area, in addition to within the field, that pollen ingestion by the larvae can represent a real hazard. Habitat types that can be found at this distance from the field include arable fields, orchards, pastures and grasslands, unmanaged strips of land along ditches or paths, field margins, fallows, scrub and even forest margins. As maize is not cropped above 800 masl, alpine butterflies were removed from the list, as well as forest species.

- Larval food plants must be commonly present in or around maize fields. The list of all the known larval food plants of each butterfly species was compared with plant inventories from maize agroecosystems.
- There must be at least some overlap between the species' larval stage and maize pollen shed (Oberhauser *et al.*, 2001), which can take place from mid June to the beginning of August in the study areas. It is the larval stage that is susceptible to *Bt*, especially the first instars (Felke *et al.*, 2002). In the case of HT maize, again the susceptible stage is mainly the larvae because it feeds off the plants although the reduction in nectar sources utilized by the adults could also affect butterfly populations. Therefore, the possible effects of HT maize will take place over a longer period of time and may affect any generation of larvae or adults.

Description of the study areas

The Bujaraloz study area is located at 327 masl in the Monegros badlands and subject to chronic droughts. It is common practice to till soil intensively for weed control and overhead irrigation is used. Maize is cropped for grain and is mostly *Bt*. The landscape is made up mostly of arable crops, fallows and scrubby non-cultivated areas.

The Almacelles study area is located in the highly intensive agricultural plains of Lleida, at 247 masl. Weed control is based on herbicide but some cultural control may be used, irrigation is overhead or flooding. Maize is cropped for grain and is mostly *Bt*. Arable crops and orchards cover most of the area leaving few uncultivated strips of land following paths and waterways and some small hills covered in grassy or scrubby dryland vegetation.

The La Seu study area is located in a plain at 691 masl in the Pyrenees. Maize is cropped for silage as part of a yearly crop rotation with winter grains so it is planted late, from May to June, and harvested in September. No-till and overhead irrigation are used with a single pre-emergence herbicide application. The maize varieties used are not *Bt* as there is no pest pressure from corn borers. The landscape is a mosaic of fodder crops, meadows, non-cultivated strips of grassland, shrub and forest.

Butterfly habitats sampled

Butterflies were sampled in three different habitats closely associated to maize: field margins, alfalfa and semi-natural vegetation.

Maize field margins included the bands of vegetation found between fields and the margins separating the field from roads or waterways. In all three study sites margins were composed mainly of herbaceous plants because it is common practice to cut or apply herbicide at least every few years for weed control.

Alfalfa is the most common field crop present in all three study areas during the growing cycle of maize and it is also the larval food plant of a number of butterfly species. In addition, when in flower it is also attractive to adult butterflies.

The third habitat sampled was the semi-natural vegetation of each area. We sampled the vegetation growing on non-cultivated land which was as similar to the natural vegetation of each area as possible. This was therefore the most variable habitat sampled. In Almacelles and Bujaraloz the potential vegetation is the *Rhamno-Quercetum cocciferae cocciferosum* plant community. Due to human disturbance the communities actually found are sparse steppe vegetation (*Ruto-Brachypodietum retusi* on north exposed slopes and *Delphinio-Lygeetum sparti* on the south slopes) which has in some locations regressed to the *Salsolo-Artemisietum herba-albae* community of halophilous plants. Finally, in La Seu, the semi-natural vegetation that can be found in the vicinity of maize fields includes a great diversity of plants both arboreus and herbaceous. The vegetation includes communities dominated by *Quercus ilex* (L.) on southern slopes; *Pinus nigra* Arnold and *Q. pubescens* Willd on northern slopes; and riverside forest in the river basin. The natural vegetation has often been altered resulting in scrubby grassland.

Field monitoring of the butterfly species

After identifying the diurnal Lepidopterans susceptible to becoming candidates for PMEM, the field monitoring was started. Here we report only the results from the first year (2012) although the field work will be continuing in the 2013 season. The monitoring should help to determine when and where the butterflies should be sampled and with what intensity.

Butterfly recording was done following the methods used by the Butterfly Monitoring Scheme (BMS) and described by Pollard & Yates (1979). Five transects were established in each study area, separated by at least 1 km. Each transect was divided into three 100 m sections. The section length was established at 100 m due to the small size of maize plots and associated habitats in the study areas. Where possible, the first section was located alongside a field margin. The second and third sections were located in the surrounding semi-natural vegetation and in alfalfa (at a distance between 5 and 25 m from the maize field). These transects were walked three times throughout the 2012 season in July, August and September. Phenology of the crops and abundance of flowering plants were recorded each time butterflies were sampled. Flora relevés were done once for each section using the Braun-Blanquet method. Butterfly nomenclature follows (García-Barros *et al.*, in press).

The Catalan Butterfly Monitoring Scheme

Unlike other European countries such as Switzerland, The Netherlands or the UK, Spain has no national butterfly monitoring scheme. Butterfly monitoring schemes are regional and only two exist: the Basque monitoring scheme (H.A.Z.I) and the Catalan Butterfly Monitoring Scheme (CBMS). Apart from these, butterfly monitoring is carried out in natural parks or as part of specific studies.

The CBMS (http://www.catalanbms.org/) is a consolidated network that has been active since 1994. Recording is done once a week from March to September by volunteers. Transect length is usually around 1.5 km. The main drawback of the CBMS is the fact that there are practically no transects in the maize growing areas, as volunteers generally prefer to monitor in natural areas.

Results and discussion

Bibliographic selection of candidates

After the bibliographic selection process the list of preliminary candidate species for PMEM in each study area was 79 species for Bujaraloz, 81 for Almacelles and 91 for La Seu. Our selection criteria were not very restrictive as it has to be taken into account that in many areas where maize is cropped most of the area is agricultural or in close contact with agricultural land, so nearly all butterflies are exposed to the pollen and therefore could be meaningful. Nevertheless, we intend to produce more restrictive selection criteria when we have all the field data at the end of 2013.

Field monitoring

Field monitoring during the 2012 season yielded a total of 30 species of butterflies from 5 families. Two species observed in La Seu were not included in the preliminary list of candidates, i.e. *Pararge aegeria* (L.) is a forest species and *Satyrium spini* (Denis & Schiffermüiller) feeds on *Rhamnus* spp., not usually found in the vicinity of maize. The remaining 28 candidate species and their abundance are shown in Table 1.

Table 1. Abundance of butterfly species in different study areas (Bujaraloz, Almacelles and La Seu) and habitats sampled (alfalfa: A, field margin: M, and semi-natural noncrop vegetation: NV). Results are expressed as number (mean±SD) of individuals per 100 m and are averages of five sites sampled in July, August and September 2012.

			Bujaraloz			**Almacelles**[1]			**La Seu**	
Species	**Family**	**A**	**M**	**NV**	**A**	**M**	**NV**	**A**	**M**	**NV**
Carcharodus alceae	Hesp.		0.1±0.1		0.1±0.1	0.1±0.1	0.2±0.1	0.1±0.1	0.1±0.1	0.1±0.1
Muschampia proto	Hesp.			0.2±0.2						
Pyrgus malvoides	Hesp.							0.1±0.1		
Spialia sertorius	Hesp.					0.1±0.1				
Thymelicus acteon	Hesp.	0.4±0.3								
Aricia agestis	Lyc.								0.1±0.1	
Celastrina argiolus	Lyc.								0.1±0.1	
Cupido argiades	Lyc.									0.1±0.1
Lampides boeticus	Lyc.	0.5±0.4			0.2±0.2		0.1±0.1	6.9±4.1	0.4±0.3	0.2±0.2
Leptotes pirithous	Lyc		0.1±0.1			0.1±0.1				
Lycaena phlaeas	Lyc.									0.1±0.1
Polyommatus icarus	Lyc.	0.9±0.6	1.8±0.8	0.8±0.3	2.0±0.7	0.1±0.1	1.7±0.4	1.5±0.9		0.2±0.2
Satyrium esculi	Lyc.									0.2±0.2
Aglais io	Nym.							0.1±0.1	0.1±0.1	
Aglais urticae	Nym.									0.1±0.1
Coenonympha pamphilus	Nym.							0.1±0.1		
Lasiommata megera	Nym.	0.2±0.1		0.3±0.3				0.1±0.1	0.1±0.1	0.1±0.1
Melanargia lachesis	Nym.								0.1±0.1	0.1±0.1
Pyronia cecilia	Nym.	0.1±0.1		0.3±0.3						0.3±0.2
Pyronia tithonus	Nym.							0.1±0.1		
Vanessa cardui	Nym.			0.1±0.1	0.1±0.1	0.1±0.1				
Iphiclides feisthamelii	Pap.							0.1±0.1		0.1±0.1
Papilio machaon	Pap.				0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	
Colias crocea	Pier.	0.7±0.3	0.4±0.2	0.4±0.2	0.3±0.2	0.2±0.2	0.3±0.3	0.7±0.2	0.3±0.1	0.5±0.2
Pieris brassicae	Pier.									0.3±0.2
Pieris rapae	Pier.	1.7±0.9	1.5±0.9	0.9±0.6	0.3±0.3	0.3±0.2		0.2±0.2		0.3±0.3
Pieris napi	Pier.	0.3±0.2	0.1±0.1	0.1±0.1	0.7±0.7	0.3±0.2	0.2±0.2	0.4±0.4	0.1±0.1	0.3±0.2
Pontia daplidice	Pier.	0.1±0.1	0.1±0.1	0.8±0.3		0.1±0.1	0.7±0.2	0.1±0.1		

[1] Almacelles is a locality in the Segrià county, close to the city of Lleida..

The greatest diversity of butterfly species was observed in La Seu (21 species) followed by Bujaraloz (13 species) and finally Almacelles (11 species). The three study areas only had 7 species in common (*Carcharodus alceae* (Esper), *Lampides boeticus* (L.) *Polyommatus icarus* (Rottemburg), *Colias crocea* (Geoffroy), *Pieris napi* (L.), *P. rapae* (L.) and *Pontia daplidice* (L.)). These species are all considered generalists and have more than one generation a year.

In Bujaraloz, of the 13 species observed, 9 were present in alfalfa, 7 in the field margin and 9 in the surrounding natural vegetation. Only *P. icarus* and the pierids *C. crocea*, *P. napi*, *P. rapae* and *P. daplidice* were common to all three habitats. In Almacelles, 8 species were found in alfalfa, 10 in the field margins and 7 in the natural vegetation. The species common to all habitats were *C. alceae*, *P. icarus*, *Papilio machaon* L., *C. crocea* and *P. napi*. Finally, in La Seu 14 species were found in alfalfa, 10 species in the field margins and 15 in the natural vegetation. The species common to the three habitats were *C. alceae*, *L. boeticus*, *Lasiommata megera* (L.), *C. crocea* and *P. napi*.

Only *C. crocea* and *P. napi* were found in all habitats in the three study areas. These two species, despite being generalists that can colonize a number of habitats and develop on different food plants, may be useful for monitoring as they would allow comparing data among sites. The use of monophagous or polyphagous species has both advantages and disadvantages. Monitoring monophagous species has the advantage that these species will be sensitive indicator species as they are dependent on one host plant and will reflect their density and abundance without much delay (Hilbeck *et al.*, 2008) and allow modelling (Perry *et al.*, 2010). On the other hand, they might over-proportionally reflect heterogeneous distribution patterns of their host plants, they may not allow to compare data from sites were the food plant may be absent and varying susceptibility of species may be overlooked. Oligophagous species could buffer this better but might be less sensitive to environmental changes (Hilbeck *et al.*, 2008).

It is interesting to observe that the highest number of species was found in the surrounding semi-natural vegetation despite the extremely arid conditions in Bujaraloz and Almacelles. It may thus be the best habitat for sampling the most meaningful species. Agricultural habitats presently contribute significantly to biodiversity in terms of species richness at the European level; however it has been signalled that agricultural intensification leads to biodiversity loss in agricultural areas. Many characteristic species of agricultural habitats figure on Europe’s red list of endangered species. Indeed, two of the species collected during 2012 (*Thymelicus acteon* (Rottemburg) and *Coenonympha pamphilus* (L.)) are listed on the European Red List of Butterflies under the category Near Threatened (Van Swaay *et al.*, 2010). In this scenario the monitoring for adverse effects of GM maize on biodiversity is essential in order to make decisions relative to nature conservation.

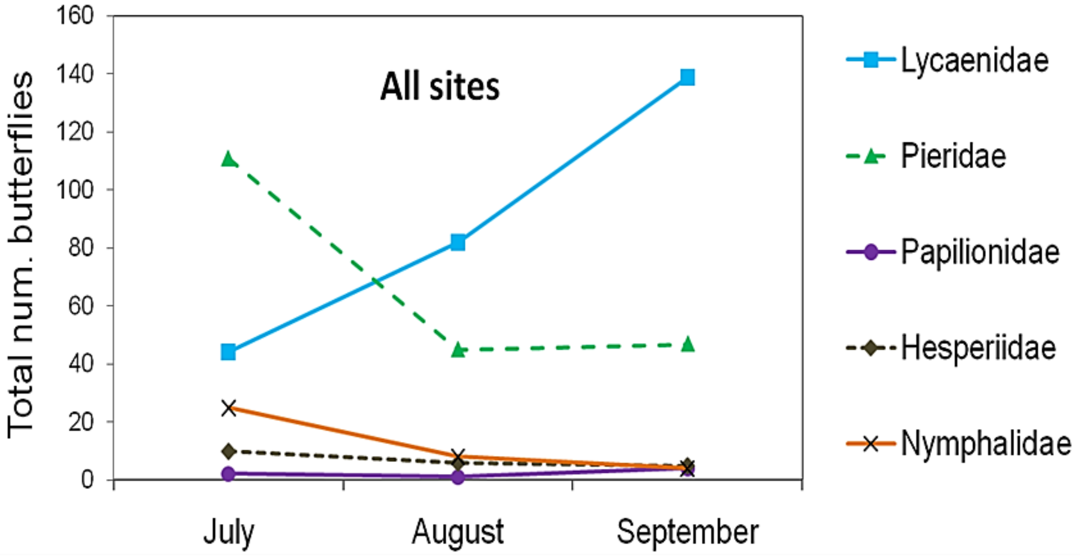


Fig 1. Abundance of butterflies at each sampling date, results are sums of all individuals recorded for a certain date.

Abundance of the different families of Lepidoptera at the three sampling dates is shown in Fig 1. The number of butterflies was highest at the beginning of the sampling period, just before maize began flowering. The number of individuals of most families declined strongly by the following sampling date in August, but recovered slightly in September. The exception was the Lycaenids, which increased throughout the season, especially in La Seu. In view of this, we started sampling earlier in 2013 with the aim of detecting the moment of maximum butterfly abundance. If butterfly numbers are higher then the number of sampling dates could be reduced.

We will continue sampling the same habitats this season (alfalfa, field margin and semi-natural vegetation). At the end of 2013 we hope to establish the most meaningful butterfly species for GS in Spain, the habitats that should be sampled, the number of sections and the number of replicates needed in order to detect variations of butterfly populations.

Adapting the Catalan Butterfly Monitoring Scheme for GS

- The CBMS has many qualities that make it a good candidate for carrying out GS for GM maize (http://www.catalanbms.org/), with some modifications:
- The number of transects in maize growing areas would have to be higher for GS but also shorter. GS to detect effects of GM maize on the environment would require high statistical power.
- Instead of weekly sampling, only few sampling dates would be required. Once the meaningful butterfly species have been selected, sampling could be done at population peaks of these species.
- GS requires long term monitoring, which could be established as part of the normal yearly monitoring activity of the CBMS.
- The information obtained from GS of GM maize regarding in Spain can be compared to that obtained in other countries by other butterfly monitoring schemes.
- The CBMS can be relied on for identification of butterfly species and knowledge of their ecology.

In addition, data from the CBMS can give a good context to understand butterfly population dynamics in maize cropping areas.

Aviron *et al.* (2009) found that it was very difficult to detect changes in butterfly populations even above 30% due to the high variability of communities and the multitude of influencing environmental factors. Therefore, environmental and other factors should be taken into account. This will require further data-mining in other pre-existing databases such as soils, altitude/latitude, climate, landscape together with the use of farm questionnaires to obtain information regarding farm management.

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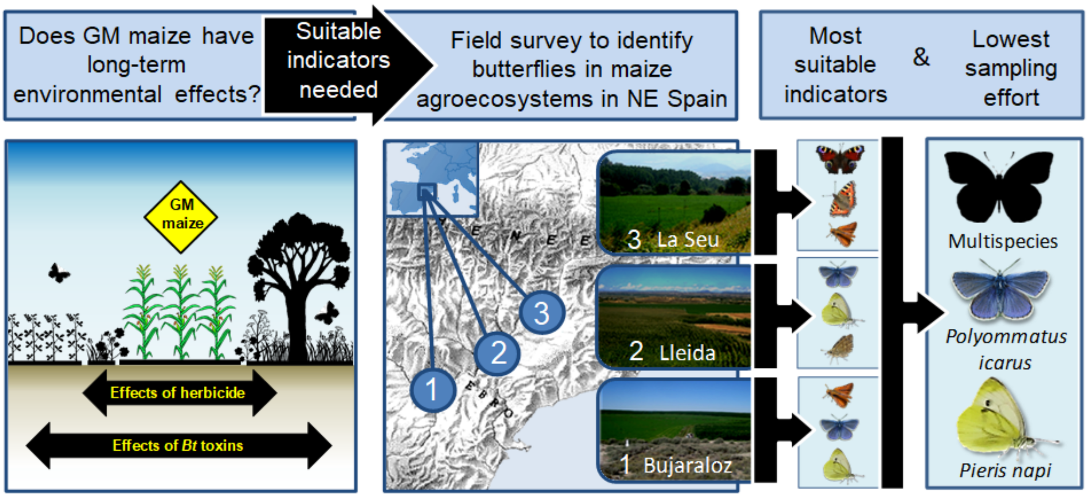
Chapter 2.

Sampling and selection of butterfly indicators for General Surveillance of Genetically Modified maize in north-east Spain



Sampling and selection of butterfly indicators for General Surveillance of Genetically Modified maize in north-east Spain

Genetically modified (GM) maize has been cultivated commercially in Spain since 1998. Although long-term environmental monitoring to detect unexpected or cumulative environmental effects (General Surveillance, GS) is compulsory in the EU, it has not been fully implemented due to the costs involved and lack of suitable methods.

The main aim of this study was to increase the feasibility of GS of GM maizes expressing insect-resistance (*Bt*) and herbicide tolerance (HT) traits.

Butterflies (Lepidoptera: Papilionoidea) were sampled using transect-counts in three differentiated maize-growing regions in north-east Spain. Five transects were established per region and sampled three times per season in two consecutive years. Transects included 100 m sections in field margins, alfalfa (Medicago sativa) and non-crop vegetation. Butterfly larvae were sampled during anthesis in maize field margins in Lleida region. Distribution of larval host plants was assessed in maize fields and neighbouring habitats in all regions.

Field data and literature were used to construct a step-by-step selection process to identify appropriate butterfly indicators for monitoring the effects of GM maize cultivation. The required sampling effort to detect effects on selected butterfly indicators was estimated by prospective power analysis.

We identified 41 butterfly species, including three protected species. Most species were potentially exposed to GM maize cultivation effects because their larval host plants were present in maize fields, margins and neighbouring habitats. We found larvae of four species in maize field margins, the most abundant were *Carcharodus alceae*. It would be possible to detect a 30% population change by sampling its host plants *Malva spp*. in 35 to 95 site pairs.

When we applied the selection procedure, the most appropriate species for monitoring depended on the region considered. However, considering sampling effort of selected indicators, for all study regions it tended to be lowest for multispecies groups (i.e. 15-32 site pairs for butterfly abundance) and for the single species *Polyommatus icarus* and *Pieris napi* (27-87 and 24-84 site pairs respectively). These indicators could be monitored through existing butterfly monitoring schemes as part of a wider environmental monitoring in agricultural regions to assess impacts of agri-environmental management.

Key words: environmental monitoring, farmland, non-target Lepidoptera, genetically engineered crops, *Bt* effects, herbicide effects.

Introduction

The cultivation of genetically modified (GM) maize (*Zea mays* L.) varieties may have effects on the receiving environment that only become apparent after widespread or long-term cultivation. For this reason, Post-Market Environmental Monitoring (PMEM) of GM plants is mandatory in the EU (EC, 2001; 2018). There are two types of PMEM (EFSA, 2011): Case Specific Monitoring (CSM) which is conducted on a case-by-case basis and focuses on potential adverse effects anticipated in the pre-release Environmental Risk

Assessment (ERA), and General Surveillance (GS) which focuses on unexpected, delayed or cumulative effects that could not be detected by the ERA; GS is compulsory for as long as the GM crop is cultivated.

Despite the fact that GS is compulsory for GM crops, the detection of effects on the environment is unlikely because currently GS is mainly based on farm questionnaires directed at the farmers (Schmidt *et al.*, 2008) and annual reviews of the scientific literature (EFSA, 2011). Recognising this shortcoming the European Food Safety Authority (EFSA), recommends using data from environmental survey networks (ESN) (EFSA, 2014) but in 2020 this has not yet been implemented. EFSA also recommends integrating GS into a wider environmental monitoring scheme. This large-scale environmental monitoring would greatly benefit our agricultural systems by informing on the effects of current agricultural practices and environmental management (Lefebvre *et al.*, 2015).

Ideally, a GS plan should be capable of detecting any potential effect resulting from GM crop cultivation (EFSA, 2011) but such a plan would require intensive monitoring that would be too costly. The most feasible option to date is a targeted GS that focuses on relevant assessment endpoints at critical moments of exposure to the GM crop cultivation. This requires taking into consideration the potential adverse effects of the cultivation of each particular GM crop and each receiving environment. Worldwide, the most widespread traits engineered into crops are insect resistance, obtained by introducing truncated *Bacillus thuringiensis* Berliner genes (*Bt* crops); and herbicide tolerance obtained by introducing genes conferring tolerance to broad spectrum herbicides (GMHT crops), (ISAAA, 2019). In this manuscript we outline a GS plan for *Bt* and GMHT traits.

The only GM crop cultivated commercially in the EU (Spain and Portugal) is *Bt* maize MON810 which expresses the Cry1Ab toxin conferring resistance to Lepidopteran stemborers. Worldwide, the deployment of *Bt* crops has been shown to be an effective control measure allowing farmers to reduce insecticide treatments (Naranjo, 2009). In Spain, MON810 has become prevalent in areas where pressure from the stemborers is high (Eizaguirre *et al.*, 2006) and in 2018 MON810 covered 115,246 ha (ISAAA, 2019), which is over a third of the Spanish maize production area (MAPA, 2020). In Spain, GM maize has been cropped for over 20 years and considerable research has focused on effects of GM maizes on non-target organisms (e.g. De La Poza *et al.*, 2005; Ortego *et al.*, 2009; Albajes *et al.*, 2012; Comas *et al.*, 2014; Arias-Martín *et al.*, 2018). However, a GS plan capable of detecting potential adverse effects of GM maize has not been fully implemented.

Butterflies (Lepidoptera: Papilionoidea) are excellent candidates for environmental monitoring because they are one of the best studied insect groups, they are good indicator organisms (Thomas, 2005), and a valued conservation goal (Van Swaay *et al.*, 2010). Most importantly, butterflies are the perfect taxa for GS monitoring given their sensitivity to *Bt* toxins and their dependence on plant species that can be altered by GMHT maize cultivation.

Maize produces large quantities of pollen that can be deposited on larval host plants in or around maize fields (Pleasants *et al.*, 2001; Lang *et al.*, 2004; Hofmann *et al.*, 2016). Lepidopteran larvae may be affected adversely when consuming *Bt* pollen, and those effects will vary in magnitude depending on the amount of *Bt* toxin ingested and the susceptibility of the species (Hellmich *et al.*, 2001; Sears *et al.*, 2001; Zangerl *et al.*, 2001; Felke *et al.*, 2002; Lang & Vojtech, 2006; Lang & Otto, 2010; Schuppener *et al.*, 2012). For this reason, butterflies have been proposed for environmental monitoring of *Bt* maize in the EU. In particular, monitoring nettle (*Urtica* spp.) feeding butterflies (*Aglais* spp.) could be a cost-effective method for monitoring environmental effects of GM maize (Lang *et al.*, 2011; Schuppener *et al.*, 2012). These nettle-feeding species have also been used as model species to study risks of *Bt* maize to non-target organisms (Perry *et al.*, 2010, 2012; Holst *et al.*, 2013; Fahse *et al.*, 2018). However, nettles are not common around maize fields in Mediterranean regions (Lee & Albajes, 2013) and therefore alternative indicators should be identified.

The deployment of GMHT maize implies a change in the herbicide regime, which modifies flora abundance and composition (Hawes *et al.*, 2003; Albajes *et al.*, 2014) in maize fields and field margins. This change in the herbicide regime may have adverse effects on butterflies that depend on host plants in maize fields and neighbouring habitats (Pleasants & Oberhauser, 2012). For this reason, some authors consider butterflies to be good indicators for monitoring GMHT maize effects (Hilbeck *et al.*, 2008). Nevertheless, weed control of GMHT maize can sometimes result in an increase of some larval host plant species compared to other weed control measures (Bigler & Albajes, 2011). In addition to plant-mediated effects on butterflies, some herbicides have been found to have direct toxic effects on non-target organisms, including Lepidoptera (Kutlesa & Caveney, 2001; Gill *et al.*, 2018; Székács & Darvas, 2018).

Butterflies unlike most arthropods, are routinely monitored by environmental surveillance networks across the EU. Therefore, appropriate butterfly indicators could be monitored through these networks as proposed by EFSA (2014).

Objectives

This study aims to outline a reliable and cost-effective general surveillance plan for monitoring the effects of *Bt* and GMHT maize cultivation on biodiversity in Mediterranean conditions. In order to achieve this, we first identify the butterflies and their host plants present in three different maize-growing regions in north-east (NE) Spain. Secondly, we use field data and literature to develop a selection procedure to identify the most appropriate species for monitoring effects of GM maizes. Finally, we estimate the sampling effort required for effect detection using selected butterfly indicators.

Materials and Methods

Study regions

The field surveys were carried out in three different maize-growing regions in NE Spain to account for variability due to differences in climate, cultural practices or landscape among others.

The regions were Bujaraloz in the Monegros Badlands (41°29′N, 0°9′O, 328 m.asl), Lleida agricultural plains (41°43′N, 0°26′E, 250 m.asl) and La Seu, in the Pyrenees (42°21′N, 1°27′E, 691 m.asl). Bujaraloz and Lleida are located in the Ebro basin in the same biogeographical region, (BSk Köppen-Geigen climate type (Kottek *et al.*, 2006)) and maize cultivation practices are similar (Farré & Faci, 2009). Maize is an important summer crop in the Ebro basin, occupying around 16% of the area of irrigated land. Pressure from Lepidopteran corn-borers is high in this region so it is common to use *Bt* varieties (MON810), whereas insecticides are rarely applied (Eizaguirre, 2012). Maize is cultivated for grain, fields are ploughed and planting takes place from March to July, pre-emergence herbicides are applied and fertilization is a mixture of mineral NPK and manure. The key difference between the agricultural regions of Bujaraloz and Lleida is that, while Lleida area has been irrigated for centuries, irrigation is fairly recent in Bujaraloz (1970s). As a result, Bujaraloz maize fields are larger (8.4 ± 1.5 ha) and landscape is composed of arable crops and large patches of native dryland vegetation and there is little arboreal cover. In contrast, Lleida landscape is that of an intensive agricultural area, maize fields are smaller (5.4 ± 0.9 ha) and arable crops and orchards cover most of the irrigated areas and there is very little semi-natural vegetation. Maize field margins in Bujaraloz and Lleida were composed of herbaceous plants because it is common practice to periodically cut, burn or apply herbicide for weed control, particularly in Lleida. Recently, intensification has reached a point that in addition to the main maize crop planted in March-April, it is increasingly frequent to plant a second maize crop in May-June, straight after harvesting winter cereals. La Seu region is very different to the other two, with cooler climate and greater rainfall (Cfb Köppen-Geigen climate type), but it is still necessary to irrigate maize. Maize is cropped for silage as part of a yearly crop rotation and agricultural practices include no-till and pre-emergence herbicide applications. *Bt* maize is not used because corn-borers are not important pests. Average maize field size was 3.0 ± 0.7 ha and landscape is a mosaic of forage crops and pastures in the valley and pastures and forest on mountain slopes. Field margins are often associated to stone walls and woody plants.

Alfalfa (*Medicago sativa* L.) is one of the most common field crops present in the study areas in summer (Madeira *et al.*, 2014; Ardanuy *et al.*, 2018; Clemente-Orta *et al.*, 2020). In Bujaraloz and Lleida, alfalfa cultivation practices are similar, the crop is grown for 4-5 years and cut around 5-6 times per season, insecticides are applied for pest control. Alfalfa management in La Seu is coordinated for all fields and cuts take place simultaneously, the crop is grown for around 5 years and cut 5 times per season, fertilization is organic and no insecticides are applied (Madeira & Pons, 2016).

Field survey of adult butterflies

A two-year survey was carried out in the three maize-growing regions in order to determine the abundance and frequency of species present around maize anthesis.

Sampling took place three times each growing season (2012 and 2013), July (maize growth stage V3-VT), August (V12-R1) and September (VT-R6). The sampling dates were chosen in order to detect the most abundant and/or frequent species at the moment of higher risk from GM maizes (namely *Bt*-maize pollen deposition). Maize stage nomenclature followed Ritchie *et al.* (1989), differing growth stages at the same sampling date were due to differences between the three study regions.

Five transects were established in each study region, separated by at least 1 km. Each transect was divided into three linear sections (100 m per section) crossing habitat types associated to maize (maize field margin, alfalfa field and non-crop areas). The section length was established at 100 m to adapt to the small size of maize plots and associated habitats in the study areas. Nevertheless, in Lleida in 2013 we increased length to 200 m sections to check if this could contribute to reduce the sampling effort.

Butterflies are rarely observed in maize fields and for this reason maize field margins, alfalfa and non-crop areas were sampled. Maize field margins included the bands of vegetation found between fields and the margins separating the field from roads or waterways (Marshall *et al.*, 1996). Alfalfa was sampled because it is an attractive nectar source and the larval food plant of various butterfly species (García-Barros *et al.*, 2013). The third habitat sampled were non-crop areas of semi-natural vegetation because maize pollen may travel considerable distances, dusting host plants of butterflies in natural areas (Lang *et al.*, 2015). Sections in alfalfa and non-crop areas were usually located at a maximum distance of 20 m from the maize field because this is the area of maximal pollen deposition (Messeguer *et al.*, 2006).

Butterflies were sampled visually by the transect method (Pollard & Yates, 1993a) used by most European Butterfly Monitoring Schemes (Schmucki *et al.*, 2016). The observer records all adult butterflies detected within a 5x5 m virtual area along a line transect, obtaining an estimate of density-abundance (for simplicity, we will use the term "abundance"). Sampling only took place when meteorological conditions were favourable for butterfly activity. Butterflies were identified to species level if possible, (Tolman & Lewington, 2011; García-Barros *et al.*, 2013) and taxonomy followed Van Swaay *et al.*, (2010).

Butterfly larval host plant study

Butterfly host plants in maize fields

Surveys of maize weeds were carried as part of a study on weed changes in irrigated crops (Juárez-Escario *et al.*, 2018). In brief, 392 maize fields were surveyed in the summer of 2009 in the Lleida province. Fields were surveyed in the counties of Segrià and Pla de l'Urgell (41º45′N, 0º36′E), Noguera (41º54′N, 0º47′E) and Pallars Jussà (42º5′N, 1º05′E). All plant species were recorded in a rectangle of 6x5 m (30 m^2), at a distance of 5 m from the field margin. An abundance-dominance score between ‘+’ and ‘5’ based on the Braun-Blanquet (1979) scale was assigned to each species. The scores were transformed into mean cover percentages (‘+’ = 0.1%, ‘1’ = 5%, ‘2’ = 17.5%, ‘3’ = 37.5%, ‘4’ = 62.5% and ‘5’ = 87.5%). Plants were identified to species using local flora (de Bolòs & Vigo, 2001) and plant nomenclature followed the International Plant Names Index (IPNI, 2020). In this study, we focused on butterfly larval host plants (García-Barros *et al.*, 2013) that were recorded in at least 5 sites.

Butterfly host plants in the vicinity of maize fields

Butterfly larvae developing on plants in GM maize field margins are highly exposed to the potential risks of *Bt* pollen drift and to the effects of changes in herbicide applications resulting from GMHT maize cultivation. It is unclear if GMHT maize cultivation would have any effects on larvae in other habitats but it is known that significant amounts of *Bt* pollen can be deposited on host plants at distances of up to 20 m from maize fields (Hofmann *et al.*, 2014). For this reason, flora relevés were carried out in maize field margins and nearby non-crop areas of semi-natural vegetation. Plants were identified within an area of 30 m^2 (dimensions ranged from 1 × 30 m in narrow margins to 5 × 6 m in alfalfa and non-crop areas) in each site. Procedure for flora identification and calculation of mean cover of larval host plants was carried out as described in the previous section.

In addition to the flora relevés, a directed survey of larval host plants was carried out in the Lleida region (details given in the following section ‘Butterfly larvae survey’).

Butterflies developing on crop plants close to maize fields could be affected adversely by GM maize cultivation. Therefore, we used literature to identify crops grown in the study areas that could be used as host plants by butterfly larvae (García-Barros *et al.*, 2013; Pujol i Palol, 2017; MAPA, 2020).

Field survey of butterfly larvae

We sampled larvae to identify which species were developing in field margins at maize anthesis and to determine the sampling effort involved (Lang *et al.*, 2011). Larvae were sampled at peak maize anthesis, in maize field margins in the Lleida region in 2013. Two separate anthesis periods were sampled according to planting date of the maize crops. The first maize crop planted around 15-March to 15-April flowers in July (henceforth anthesis

I, n = 10), the second maize crop planted around 15-May to 15-June following harvest of winter cereal, flowers in August (henceforth anthesis II, n = 12). At each site, two field margins were surveyed (except in two sites were only one margin was sampled). In each margin 100 linear meters were searched, considering a width of one metre. In total 4,200 linear meters were surveyed, equivalent to an area of 4,200 m^2. Only larval host plants (García-Barros *et al.*, 2013) of the butterflies identified in the field survey were sampled. The preferred sampling methodology was visual inspection, but in some cases, frappage was applied because it was more suitable for sampling grasses and thorny plants, similar as described by Lang *et al.*, (2013). To quantify sampling effort, we recorded plant number, dimensions and sampling time. All Lepidoptera larvae (butterflies and moths) were collected, reared at the laboratory to adult and identified to species, if possible, following literature (e.g. Rougeot & Viette, 1980; García-Barros *et al.*, 2013).

Selection criteria for butterfly indicators

We developed and applied a step-by-step selection process to identify the most suitable species for monitoring effects of GM maize cultivation in NE Spain. Selection criteria were based on similar studies (e.g. Schmitz *et al.*, 2003; Hilbeck *et al.*, 2008).

In the first place, all species that were not present in at least two of the sites sampled per region per year were excluded. Following this step, further exclusion and prioritisation criteria were applied to the candidate species, explained in detail as follows:

A. Exclusion criteria.

   1. Exposure. The first consideration for selecting species for field monitoring was the exposure of butterfly larvae to *Bt* maize pollen and/or direct and indirect exposure to herbicides. Species could be excluded if they were not exposed to *Bt* toxins and could not be affected by changes in herbicide regimes: a) species with no overlap between the larval stage and maize anthesis; b) species not exposed to *Bt* pollen due to endophytic or below-ground larval stage; or c) species whose host plants are not usually found in the vicinity (<20 m) of maize fields.

   2. Sensitivity. This can refer to the sensitivity of the species to to *Bt* toxins expressed in the plant or the pollen (Felke *et al.*, 2002, 2010; Lang & Vojtech, 2006; Kjær *et al.*, 2010; Lang & Otto, 2010; Schuppener *et al.*, 2012) or to

   direct or indirect effects of modified herbicide regimes (Pleasants & Oberhauser, 2012). Species insensitive to glyphosate-based herbicides and *Bt* toxins were excluded.

3. Responsiveness. Species selected for monitoring should reflect changes of the system. For this reason, we decided to exclude crop pests or species with a strong migratory behaviour in the study areas (Stefanescu *et al.*, 2011b) because it is difficult to establish causal effects between fluctuations in the species' abundance and changes at the local scale.

A. Prioritisation criteria. In most cases there was insufficient information regarding the direction of the possible impacts of GM maizes on butterflies so we applied ranking criteria to select the candidates most appropriate for monitoring. Following the exclusion process, protected species (Van Swaay *et al.*, 2010) and maize-feeding species (critically exposed to *Bt* toxins) were reincorporated as potential candidates. The following aspects were considered:

   1. Exposure. Species most exposed to potential risks were ranked positively. If the species was recorded in both consecutive years we assigned the species one point; if the species' host plant was present in or around maize fields we assigned one point; if we detected larvae of the species during the larval survey, we also assigned the species one point; species feeding on maize were also given one point.

   2. Sensitivity. If the species was known to be sensitive to *Bt* toxins or to herbicide, according to literature, we gave the species one point.

   3. Responsivenes. We selected species' ecological or biological attributes that could make them more suitable for monitoring. a) Species' mobility because we expect less mobile species to be the most impacted by onsite changes (Hilbeck *et al.*, 2008; Aviron *et al.*, 2009), we assigned mobility classes according to literature (Stefanescu *et al.*, 2011a), giving sedentary species one point and low mobility species 0.5 points. b) Habitat preferences, open-habitat species were given 0.5 points because we would expect them to better reflect changes in areas dominated by arable agriculture.

4. Conservation value. Protected species according to the European IUCN red list (Van Swaay *et al.*, 2010) or the Spanish red list (Verdú *et al.*, 2011) were prioritised by giving one point.
5. Availability of information on the species' distribution, fluctuations in abundance, ecology or biology. The biology and ecology of species used as indicators should be well known. For instance, a) we gave one point to species used as indicators for monitoring change in agricultural systems in the EU, i.e. European grassland indicator species (EEA, 2013) or species used for evironmental monitoring of *Bt* maize (Perry *et al.*, 2010, 2012; Holst *et al.*, 2013); b) we ranked the availability of information on each species by using the fraction of bibliographic references available on the Web of Science (WOS) divided into the highest total number of references for any of the species The search was done using the accepted name of the species and its previous taxonomic synonims.

Construction of multispecies indicators

When butterfly species pools' are used for monitoring, instead of single species, this usually increases statistical power, resulting in a reduction of the sampling effort required for effect detection (Lang, 2004; Lang & Bühler, 2012; Lang *et al.*, 2019). For this reason, we aggregated* single species into multispecies indicators; indicator composition is given in the supplementary information (Appendix **A**). The individual species integrating each multispecies indicator could vary between sites: the indicator 'all species' resulted from calculating the abundance of all butterflies recorded in any given site; "migrant species" aggregated any migrant species recorded in a given site (Stefanescu *et al.*, 2011b); "mobile species" aggregated any species with high dispersion capacity (Stefanescu *et al.*, 2011a); "low mobility species" aggregated any species with low dispersion capacity (sedentary and low mobility species); "open habitat species" aggregated species linked to open habitats (from Herrando *et al.* 2016); "grassland indicators" aggregated any of the European grasslands indicator species (EEA, 2013). Finally, species number was included because it allows to further reduce sampling effort (Lang & Bühler, 2012).

*Note: In order to construct multispecies indicators, all species assigned to the same group were summed for each sampling date and site and then calculations were performed the same as for single species.

Prospective power analysis

Prospective power analysis was carried out to estimate the sample sizes (always expressed as number of pairs of sites where GM *vs*. non-GM crops are grown) needed to detect a change in butterfly populations between GM and non-GM sites using an unpaired two sample *t*-test (Perry *et al.,* 2009). The probability of committing a type I error (α) was set at 0.05 and type II error (β) was set at 0.2, (statistical power =0.8). The statistical power measures the chance of detecting an effect of a known magnitude using a specified experimental design. The effect size was established as a 30 % change regarding the comparator population (non-GM sites), considered adequate for this type of studies. Comparator populations were approximated by calculating average annual butterfly abundance in each region. Data were transformed by $\log_{10}$ (x+1) for normalization and power was calculated with the (JMP Pro®) software.

Results

Field survey of adult butterflies

A total of 41 butterfly species were recorded during the field survey of maize agroecosystems in NE Spain (Table 1); the dataset is available at Mendeley Data (Lee, 2020a). We detected three protected species, according to the EU Red List (Van Swaay *et al.*, 2010): *Carcharodus flocciferus* (Zeller), *Hipparchia fagi* (Scopoli) and *Thymelicus acteon* Rottemburg. Butterfly abundance (including identified and unidentified specimens) was high in all regions, 62.4 ± 38.2 butterflies/km (mean ± standard deviation, SD). In Bujaraloz, abundance was 79.4 ± 40.5 butterflies/km and 16 species were recorded, three species represented 64% of the counts (*Pieris rapae* (L.), *Polyommatus icarus* (Rottemburg) and *Pieris napi* (L.)). In Lleida, butterfly abundance was 46.8 ± 24.6 butterflies/km and 15 species were detected; similarly to Bujaraloz, the same three species represented 64% of the total counts. Bujaraloz and Lleida shared almost all species, with three exceptions: *Pararge aegeria* (L.) was not recorded in Bujaraloz where there is no arboreal cover; *T. acteon* was frequent in Bujaraloz (present in 40 % of the samplings) but not recorded in Lleida; and finally *Gegenes nostrodamus* (Fabricius), one of the few butterflies that can feed on maize, was recorded only in Bujaraloz. In La Seu d’Urgell, butterfly abundance was 61.1 ± 43.3 butterflies/km and 37 different species were recorded.

Twelve species were shared across the three regions; but only seven species were present in at least 50 % of the counts across all regions (Table 1): the Lycaenidae *Lampides boeticus* (L.) and *P. icarus*; (L.), the Nymphalidae *Lasiommata megera* (L.), and the Pieridae *Colias crocea* (Geoffroy), *P. napi*, *P. rapae* and *Pontia daplidice* (L.).

Table 1. Adult butterflies (Lepidoptera: Papilionoidea) recorded in maize agroecosystems in NE Spain. Mean (m) number of butterflies per km and standard deviation (SD) were calculated by averaging the mean number of butterflies recorded per season in each site (5 sites per region), %Fr is the proportion of site x year combinations where the species was present. Three protected species were detected (Near threatened (NT) status according to the IUCN red list (Van Swaay *et al.* 2010).

Family/group	**Species**	**IUCN**	**Bujaraloz**		**Lleida**		**La Seu**	
			m ± SD	**%Fr**	**m ± SD**	**%Fr**	**m ± SD**	**%Fr**
Hesperiidae	*Carcharodus alceae* (Esper)		1.2 ± 2.1	40	1.3 ± 2.1	50	0.4 ± 0.8	30
	Carcharodus baeticus (Rambur)		0.1 ± 0.4	10				
	Carcharodus flocciferus (Zeller)	NT					0.2 ± 0.7	10
	Gegenes nostrodamus (Fabricius)		0.1 ± 0.4	10				
	Muschampia proto (Ochsenheimer)		0.2 ± 0.7	10				
	Pyrgus malvoides (Elwes & Edwards)						0.3 ± 0.7	20
	Spialia sertorius (Hoffmannsegg)				0.1 ± 0.4	10		
	Thymelicus acteon Rottemburg	NT	1.8 ± 2.7	40			0.1 ± 0.4	10
	Thymelicus lineola (Ochsenheimer)						0.1 ± 0.4	10
Lycaenidae	*Aricia agestis* (Dennis &						0.1 ± 0.4	10
	Celastrina argiolus (L.)						0.1 ± 0.4	10
	Cupido argiades Pallas						0.2 ± 0.5	20
	Lampides boeticus (L.)		2.0 ± 3.0	60	0.8 ± 1.1	50	15.0 ± 33.6	90
	Leptotes pirithous L.		0.4 ± 0.6	40	0.1 ± 0.3	10	0.3 ± 0.7	20
	Lycaena phlaeas (L.)						0.3 ± 0.5	30
	Polyommatus icarus (Rottemburg)		11.8 ± 11.7	100	10.3 ± 6.3	100	4.8 ± 6.7	80
	Satyrium esculi (Hübner)						0.3 ± 1.1	10
	Satyrium spini Dennis &						0.1 ± 0.4	10
Nymphalidae	*Aglais io* L.						1.4 ± 2.5	50
	Aglais urticae (L.)						0.2 ± 0.5	20
	Coenonympha pamphilus (L.)						0.7 ± 0.9	40
	Hipparchia fagi (Scopoli)	NT					1.0 ± 1.8	40
	Lasiommata megera (L.)		1.7 ± 2.4	60	0.6 ± 1.2	20	1.6 ± 1.3	70
	Maniola jurtina L.						0.7 ± 1.1	40
	Melanargia lachesis Hübner						0.8 ± 0.9	50
	Melitaea didyma Esper						0.1 ± 0.4	10
	Pararge aegeria (L.)				0.1 ± 0.4	10	1.9 ± 2.3	60
	Polygonia c-album (L.)						0.3 ± 0.5	30
	Pyronia bathseba (Fabricius)						0.1 ± 0.4	10
	Pyronia cecilia (Vallantin)		2.8 ± 3.8	60	1.3 ± 2.3	30	1.1 ± 2.1	30
	Pyronia tithonus (L.)						0.8 ± 1.2	40
	Vanessa atalanta (L.)						0.1 ± 0.4	10
	Vanessa cardui (L.)		0.1 ± 0.4	10	1.8 ± 4.1	50	0.9 ± 1.4	40
Papilionidae	*Iphiclides feisthamelii* (Duponchel)						0.2 ± 0.5	20
	Papilio machaon L.				1.3 ± 1.9	40	0.2 ± 0.5	20
Pieridae	*Colias crocea* (Geoffroy)		9.5 ± 8.7	100	3.9 ± 3.5	70	4.1 ± 2.2	100
	Gonepteryx rhamni (L.)						0.1 ± 0.4	10
	Pieris brassicae (L.)		0.6 ± 1.2	20	0.4 ± 1.1	20	2.1 ± 1.8	70
	Pieris napi (L.)		10.8 ± 9.5	80	12.7 ± 13.9	90	5.6 ± 4.6	100
	Pieris rapae (L.)		28.2 ± 20.0	100	6.8 ± 7.0	90	7.7 ± 6.0	90
	Pontia daplidice (L.)		2.2 ± 2.1	70	1.9 ± 2.0	70	0.3 ± 0.5	30
Papilionoidea*	**All**		**79.4 ± 40.5**	**100**	**46.8 ± 24.6**	**100**	**61.1 ± 43.3**	**100**

*Papilionoidea includes both identified species and butterflies not identified to species.

There appeared to be few differences according to the habitat sampled (Appendix B). A higher number of species tended to be recorded in the non-crop areas followed by field margins. Conversely, abundance in different habitats appeared to depend on the region considered. Although the affinity of the butterfly species for each habitat was not analysed, some species were recorded across all habitat types in all regions, such as *Carcharodus alceae* or *P. icarus* whereas there were some species which were frequently found in non-crop areas but rarely in maize field margins or alfalfa, such as *Pyronia cecilia* (Vallantin) and *Pontia daplidice* (L.). In alfalfa, the Lycaenidae *L. boeticus* and *P. icarus*, and the Pierids C. *crocea*, *P. napi* and *P. rapae* were abundant in all regions, particularly when the alfalfa was flowering.

Larval host plant study

Butterfly host plants in maize fields

Weed cover is usually low in maize during summer but 33 species from 10 plant families were recorded. The most abundant and frequent plant in maize fields was *Abutilon theophrasti* (Medik), Malvaceae, which is a larval host to *C. alceae*. This plant was recorded in 24% of the 392 sites sampled and its mean cover was 2.5 ± 10.8 % (SD). The other frequent weeds belonged in families Poaceae, Asteraceae, Polygonaceae, Brassicaceae, Rosaceae, Malvaceae and Plantaginaceae (Appendix C). Overall, the weeds recorded in maize fields can host larvae of 20 butterfly species according to literature (García-Barros *et al.*, 2013).

Butterfly host plants in the vicinity of maize fields

The larval host plants of most of the butterflies were recorded mainly in field margins (Appendix C), dataset available at Mendeley Data (Lee, 2020b). There were also larval food plants in the non-crop areas. In some cases, this was the only place where larval host plants were recorded. For instance, *P. cecilia*, feeds on the grass *Brachypodium* spp. a plant native to the arid landscapes of Bujaraloz and Lleida.

In addition, we identified 25 crops that can be used as larval host plants across the study regions.

Butterfly larvae survey

In total, 4200 m^2 of margins were sampled in 22 sites in the Lleida region. In July (Anthesis I), 19 margins were sampled across 10 sites and 1,034 butterfly food plants were searched, finding 49 butterfly larvae and 35 moth larvae. In August (Anthesis II), 23 margins were sampled in 12 sites, 774 host plants were searched and 60 butterfly larvae and 41 moth larvae were collected. Although 34 different plant genus or species were sampled, butterfly larvae were only found on four plant species (dataset available at Mendeley Data (Lee, 2020c)). *C. alceae* and *Vanessa cardui* (L.) were found on *Malva sylvestris* (L.); *Papilio machaon* L. on *Foeniculum vulgare* Mill; and *Leptotes pirithous* (L.) on alfalfa. More Lycaenidae larvae were collected from alfalfa and *Ononis spinosa* (L.) but larvae died so it was not possible to identify the species. Various moth species were also recorded; the most abundant were found on *M. sylvestris*: *Acontia lucida* (Hufnagel) (Noctuidae) and *Pardoxia graellsii* (Feisthamel) (Nolidae).

The most abundant butterfly larvae were *C. alceae* and *P. machaon*. The host plant of *C. alceae*, *M. sylvestris,* was found in 91 % of the 22 sites sampled. A total of 23 larvae were collected in July and 57 larvae in August; the searching time to find one larva on *M. sylvestris* in any given margin was 9.7 minutes in July and 3.6 minutes in August (Table 2) According to prospective power analysis, it would be necessary to sample 35 to 95 site pairs (GM *vs*. non-GM site pairs) to detect a 30% difference in abundance of *C. alceae* larvae between sites (α =5, β =0.8). In the case of *P. machaon*, the host plant *F. vulgare* was recorded in 64 % of the 22 sites; 17 larvae were collected in July but only 2 small larvae were collected in August. This means that the time to find one larva on *F. vulgare* in any given margin was 5.2 minutes in July but it rose to 26.8 minutes in August (Table 2). According to prospective power analysis, it would be necessary to sample 62 to 787 site pairs (GM *vs*. non-GM site pairs) in order to detect a 30% difference in abundance of *P. machaon* larvae between sites (α =5, β =0.8).

Table 2. Butterfly larvae recorded in maize field margins in the Lleida region. The table shows the plant species on which butterfly larvae were found. Mean abundance, standard deviation (SD) and frequency (%Fr) of the butterfly larvae is given per site. Larvae were sampled during the flowering period of maize crops: July (anthesis I, 10 sites) and August (anthesis II, 12 sites). The sampling effort is shown as the number of minutes required to find a single larva at any given site.

		Anthesis I						**Anthesis II**					
		larvae			**host plant**			**larvae**			**host plant**		
Host plant	**Butterfly**	**mean**	**SD**	**%Fr**	**Mean cover (%)**	**SD**	**Effort (min/larva)**	**mean**	**SD**	**%Fr**	**Mean cover (%)**	**SD**	**Effort (min/larva)**
Foeniculum vulgare	*Papilio machaon*	1.6	2.9	30	2.2	1.6	5.2	0.3	0.7	8	2.8	4.5	26.8
Malva sylvestris	*Carcharodus alceae*	1.2	1.5	40	1.7	1.7	9.7	2.9	5.6	58	1.6	1.9	3.6
	Vanessa cardui	0.1	0.3	10			101.5	0.1	0.2	10			144.6
Medicago sativa	*Leptotes pirithous*	0.1	0.2	10	15.8	7.8	221.4	0.8	1.0	20	1.0	1.5	3.4
	Lycaenidae sp.	0.3	0.5	20			55.4	0.1	0.2	8			39.6
Ononis spinosa	*Lycaenidae sp.*	0.8	1.0	20	3.6	2.2	18.7	0	.	.	0.5	0.3	.

Selection of butterfly indicator species

From the initial 41 butterfly species, 25 species were recorded in at least two sites in any given region per year. After applying the exclusion criteria (Fig. 1), we excluded eight species because they are migrants in the study area. Two of the migrants were also crop pests, and another migrant had an endophytic larval stage. Thus, we were left with 17 candidate species. At this stage, we reincorporated the protected species and the single maize-feeding species to the list of candidates. This left seven species in Bujaraloz, six in Lleida and 18 in La Seu.

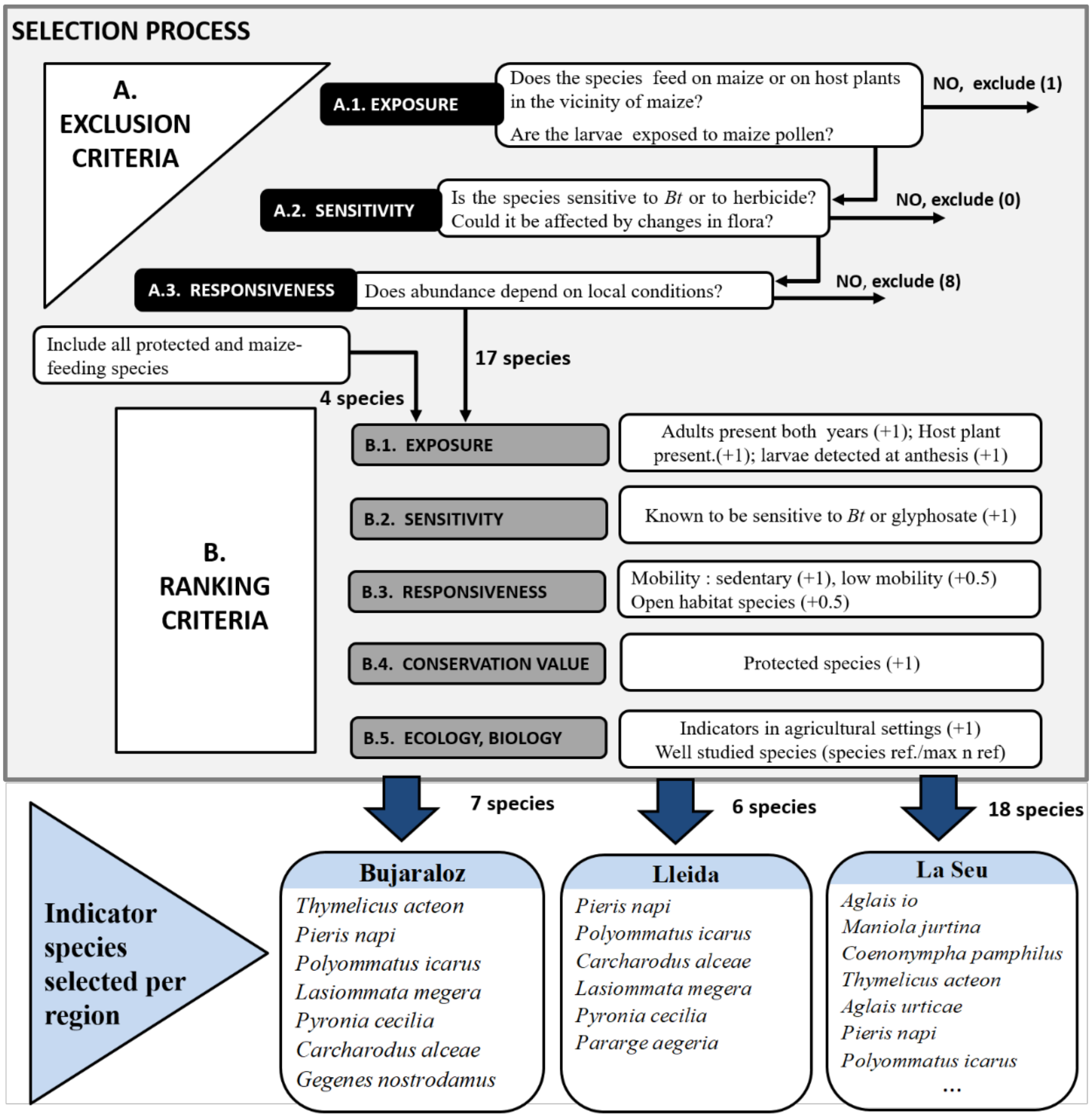


Fig. 1. Flowchart depicting the process followed to select suitable butterfly species for monitoring effects of GM maize and the final list of the highest ranking six candidates selected per region. The selection process was only applied to the 25 species present in at least two sites per year.

We applied the ranking process to select the most suitable species for GS of GM maize and then we selected the six/seven candidates with the highest rank per region (Appendix D). For Bujaraloz and Lleida most candidates were shared (*P. icarus*, *P. napi*, *L. megera*, *P. cecilia* and *C. alceae*) although the order differed between regions (Fig. 1). There were two exceptions: in Bujaraloz *T. acteon* was one of the most suitable indicators (with the highest score) but this species was not recorded in Lleida. Similarly, in Lleida *P. aegeria* was one of the most suitable species but it was not recorded in Bujaraloz. Conversely, in La Seu, the six most suitable species were nettle butterflies *Aglais io* L. and *Aglais urticae* (L.), grassland indicators *T. acteon*, *Maniola jurtina L.*, and *Coenonympha pamphilus (L.)* and *P. napi*. The only candidate species common to all three regions were *P. napi and P. icarus*.

Prospective power analysis of butterfly indicators

Prospective power analysis was applied to the selected indicator species for GS of GM maize and to the multispecies groups. The sampling effort required to detect a 30% population change of the indicators was very variable for the different butterfly indicators across regions and years (Table 3). Regarding single species, required sampling effort (number of GM *vs* non-GM site pairs) would be lowest for *P. napi* and *P. icarus* across the three regions. If multispecies pools were used for monitoring, it would be possible to reduce sampling effort. For instance, it would be possible to detect a 30% decrease in butterfly abundance by monitoring 17-32 site pairs and a 30% decrease in species' number by monitoring 7-27 site pairs.

One way to reduce the number of required sampling sites is through increasing transect length, for this reason we used 300 and 600 transects in Lleida in year 2 but this did not appear to reduce sampling effort (Table 3).

Table 3. Sampling effort, in number of site pairs, needed to detect a 30% change in butterfly abundance or species number between GM *vs* non-GM maize fields in each region. The sampling effort was calculated by prospective power analysis ($\alpha = 0.05$ and $\beta = 0.2$) on transformed data ($\log_{10}(x+1)$).

	Bujaraloz		**Lleida**			**La Seu**	
Butterfly indicator	**Year 1**	**Year 2**	**Year 1**	**Year 2**	**Year 2***	**Year 1**	**Year 2**
Species							
Aglais io						394	126
Aglais urticae							278
Carcharodus alceae	567	103	126	191	232	222	567
Coenonympha pamphilus						567	113
Lasiommata megera	191	110		185	165	240	22
Maniola jurtina							87
Pararge aegeria				567	143	567	40
Pieris napi	84	24	68	37	34	54	32
Polyommatus icarus	54	35	27	42	41	81	87
Pyronia cecilia	185	68		80	62	319	242
Thymelicus acteon	133	191					567
Multispecies groups							
Papilionoidea (all species)	26	17	15	23	21	32	22
Migrant species	27	21	25	28	25	42	20
Mobile species	41	16	24	30	28	31	22
Low mobility species	64	44	567	80	58	30	29
Open habitat species	41	25	21	29	41	47	35
Grassland indicator species	41	24	19	43	42	72	33
Species' number	27	7	13	19	17	9	13

*In Lleida, 300 and 600 m transects were used in 2013.

Note: some species were not observed in some regions and years so there is no data.

Discussion

Adult butterflies across maize agroecosystems

Butterflies were abundant around maize anthesis in all study regions, including the arid landscapes around Bujaraloz and the intensive agricultural region of Lleida. This finding contrasts to the belief that butterflies are not common in the maize agroecosystems in Spain. For instance, the mathematical models developed to assess risks of Cry1Ab from MON810 to non-target Lepidoptera in the EU (Perry *et al.*, 2010), did not include any Lepidoptera representative for Spain, because Lepidoptera were not considered to be common during maize anthesis. However, when conducting butterfly surveys close to maize fields, other authors also found butterflies to be relatively abundant and diverse in maize agroecosystems, particularly in field margins (e.g. Wallis de Vries *et al.*, 2017; Lang *et al.*, 2019b).

There were considerable differences between the species present in different maize-growing regions, particularly between the Pyrenees (La Seu) and the Ebro basin (Lleida and Bujaraloz). These differences highlight the need to perform field surveys in all receiving environments. Most differences could be expected considering the differences between study regions, but there were exceptions. For instance ,.*T. acteon* was rather frequent in Bujaraloz but absent from Lleida, the two most similar regions in terms of climate and management.

Few non-migrant species were widespread in all three regions, evidencing the difficulty in selecting single species as indicators even across a relatively small geographical scale. One way around this can be the use of different indicator species according to biogeographical regions. The peacock butterfly (*A. io*) was relatively common in the Pyrenees so it could be used as an indicator species to monitor potential impacts of GM maize, as proposed by other authors (Perry *et al.*, 2010; Holst *et al.*, 2013; Arpaia *et al.*, 2018). Conversely, in the more arid regions species suitable for monitoring effects of GM maize could be *P. icarus, P. napi or T. acteon*.

Distribution of larval host plants

When selecting butterflies for environmental risk assessment it is essential to determine the distribution of their larval host plants in the maize agroecosystem. As this will allow to infer the exposure of butterfly species to risks from the cultivation of GM maize. Weeds are not abundant within maize fields due to herbicide spraying, hence butterfly larvae would use mostly field margins where larval host plants are abundant (this study and Pywell *et al.*, 2004; Lang *et al.*, 2013; Arpaia *et al.*, 2018; Wallis de Vries *et al.*, 2017). For instance, in the US, at the beginning of field deployment of *Bt* maize there was considerable concern that this crop could have adverse effects on the monarch butterfly populations (Pleasants *et al.*, 2001; Sears *et al.*, 2001; Stanley-Horn *et al.*, 2001; Anderson *et al.*, 2005). Nevertheless, it was found that it was unlikely that significant harm could occur in the field due to the relatively low exposure of monarch larvae (Anderson *et al.*, 2004) and low sensitivity to most *Bt* toxins (Perry *et al.*, 2012). However, when GMHT maize became widely cultivated across the US corn belt, increasing glyphosate treatments reduced larval host plants which was linked to the decline of the monarch population. In this regard, it was only possible to link GMHT maize cultivation with monarch population decrease because there was information regarding the decrease in abundance of the larval food plant.

Risks to butterflies in non-crop areas

In the Mediterranean region maize tends to be grown in intensive agricultural settings where non-crop areas are relatively small (Ardanuy *et al.*, 2018; Clemente-Orta *et al.*, 2020). The pressures on specialist dry grassland butterfly populations are particularly strong because irrigated agriculture results in the perturbation, reduction and fragmentation of their natural habitats. In this regard, some butterflies detected in this study would depend exclusively on host plants present in the strips of natural vegetation present in non-crop habitats. For instance *P. cecilia* was very rarely found out of the non-crop areas where its food plant *Brachypodium retusum* grows. Another example is *T. acteon;* its absence in Lleida could be explained by the low proportion of suitable non-crop habitats due to the agricultural intensification in this area. *T. acteon* is a specialist in dry calcareous grasslands and has been known to disappear from areas where intensive monocultures are established because the species requires large patches of host plants (García-Barros *et al.*, 2013).

Monitoring butterfly larvae

We recorded 15 butterfly species as adults in the Lleida region but we only found larvae of four of those species. The diversity and number of the butterfly larvae was low considering the relatively high sampling effort invested (1,808 butterfly host plants were sampled across 22 sites). Sampling Lepidoptera larvae is usually time-consuming compared to sampling adult butterflies (Lang *et al.*, 2011) and low numbers are obtained (Gathmann *et al.*, 2006b) unless species are particularly abundant or easy to detect (Lang *et al.*, 2011). The reasons for this low success of larval sampling can be due to factors that make larvae hard to spot; larvae are often cryptic or may only be active in certain conditions. For instance *L. megera* has nocturnal larvae and may be hidden during the day.

Different strategies can be used to reduce the sampling effort needed to detect larvae, such as mapping larval host plants and using a clear sampling strategy. For instance, Lang *et al.* (2013, 2011) proposed the survey of nettle stands around the maize fields as a cost-effective sampling strategy. However, this recommendation is not applicable to arid Mediterranean conditions where nettles and their associated butterfly species are not common in the vicinity of maize fields. In our conditions, we found that *Malva* spp. occurred in all our study areas and it could be a promising group of plants to sample. Larvae of *C. alceae* larvae were abundant and easy to spot in the field as they fold leaves of *Malva spp*. In addition, other Lepidoptera larvae were found on this plant including the butterfly *V. cardui* and the moths *A. lucida* and *P. graellsii*. Monitoring this plant and the larvae that it hosts could represent a cost-effective option for assessing the effects of GM maize on biodiversity in the Lleida region. Finally, although the larvae of *P. machaon* were relatively abundant on *F. vulgare* this plant has filiform leaves that would not retain *Bt* maize pollen.

Selection of indicator species

In this study we constructed and applied a selection process to identify species appropriate for GS of GM maize in three differentiated maize-growing regions in NE Spain. The selection process was based on the potential risks and the pathways through which they could be realised, similar to other authors (Schmitz *et al.*, 2003; Van Wyk *et al.*, 2007; Hilbeck *et al.*, 2008) but we also included the capacity of the butterfly species to reflect impacts of GM maize. This is a highly relevant aspect that has often been overlooked in other studies. For instance, migrant species and pest species tend to be very abundant in farmland and are therefore suitable for statistical analysis (Comas *et al.*, 2013). However, linking measured differences in butterfly populations to the effect of GM crop cultivation (EFSA, 2011) can be difficult because pest species' abundance can depend on the host crop area and pest management. In the case of migrant species, their abundance may be linked to conditions at their place of origin (Stefanescu *et al.*, 2011b).

In our selection process, the potential exposure of butterflies to impacts from GM maize cultivation was assessed using real data on the distribution of adult butterflies and their larval host plants in different habitats of the maize agroecosystem. One of the most relevant results of this selection process was the clear differences in candidate species between the three maize-growing regions studied. The nettle butterflies *A. io* and *A. urticae* were selected for GS of GM maize in La Seu,, in agreement with the numerous studies that focus on this species for the risk assessment of *Bt* maize in the more humid EU (Perry *et al.*, 2010; Holst *et al.*, 2013; Arpaia *et al.*, 2018; Fahse *et al.*, 2018; Leclerc *et al.*, 2018). In contrast, in more arid areas, butterfly species appropriate for GS of maize were *P. icarus*, *C. alceae* or *P. napi*.

The process we developed for indicator selections is broadly applicable to any Mediterranean maize-growing region but it would be necessary to survey butterflies and their host plants in all receiving environments.

Sampling effort for effect detection

This study focused on selecting butterflies that could be most critically exposed and monitored with the lowest sampling effort, either in the field or using data from butterfly monitoring schemes, as recommended by EFSA (2014). A feasible monitoring plan requires a compromise between important qualities of the indicator such as exposure or conservation value and requirements for statistical analysis (abundance, sample size). Hence, after applying the selection criteria we used prospective power analysis to estimate the required sampling effort of the indicators. Sampling effort was consistently lowest for *P. icarus* and *P. napi* in all regions considered. On one hand, *P. icarus* could be suitable for monitoring GM maize effects because it is widespread and common in European farmland (EEA, 2013), it does not have migrant populations and it is not a pest species. On the other hand, this species feeds on many Fabaceous crops so its abundance can depend on the distribution and management of those crops. Therefore, it could be preferable to select *P. napi* as this species does not feed on any crops in the study area and the required sampling effort is acceptable (monitoring 24 to 84 site pairs would allow to detect a 30% population decline due to GM maize). Other candidate species would require a higher sampling effort, for instance *T. acteon* in Bujaraloz (133-191 site pairs), *P. cecilia* in Lleida (62-80 site pairs) or *Aglais io* in La Seu (126-394 site pairs).

When designing GS it is important to take into account the effort required to detect effects should they occur. In this regard, the best option would be to monitor species' pools as they allow to gain in statistical power (Lang & Bühler, 2012; Lang *et al.*, 2016). As expected, the sampling effort to detect a 30% decrease was lowest for species number (7-27 site pairs) but it would also be feasible to monitor abundance of multispecies groups, such as mobile species (16-41 site pairs). Monitoring the grassland indicator group would require a higher sampling effort (19-72 site pairs) but the use of this groups has considerable benefits. They are already surveyed for monitoring change in agricultural environments across Europe (EEA, 2013). Their biology, ecology and population trends are increasingly well known so that GS of maize could be integrated into a community-wide monitoring plan, for instance to assess the effects of the measures implemented through the EU's Common Agricultural Policy (CAP) (Lefebvre *et al.*, 2015).

Sampling effort in terms of number of sites could be further reduced by increasing transect length or sampling frequency (Brereton *et al.*, 2011; Lang & Bühler, 2012). Transect length and sampling frequency used in this study were rather low compared to other studies (Lang *et al.*, 2013, 2016, 2019). However, Lang and Bühler (2012) found that using transects of a similar length to those in our study (300 m) and the same sampling frequency (3 visits) could still capture around 70% of the species present.

Could Environmental Survey Networks (ESNs) be used for GS of GM maize?

In north-east Spain there is a long-term butterfly monitoring network, the Catalan Butterfly Monitoring Scheme (CBMS). This network has been recording butterflies in the region since 1998 and this network could be suitable for monitoring the effects of GM maize cultivation on butterflies. In a previous study, we found that small changes in butterfly abundance could be detected between transects located in agricultural areas compared to transects located in areas with less agricultural prevalence (Lee *et al.*, 2020). There were similarities between the results of the CBMS study and this one. For instance, the 14 most widespread butterfly species in the maize agroecosystems were among the 50 most widespread species in a 15-year dataset from the 11 diverse agricultural transects distributed across NE Spain (Lee *et al.*, 2020). There were also similarities regarding the best indicator species for effect detection; the most sensitive species in farmland were *L. megera*, *L. phlaeas*, *P. aegeria* and *P. icarus*. Conversely, a change in *P. napi* abundance could only be detected if multiyear (at least 5 years) data was used (Lee *et al.*, 2020). Clearly, data from the CBMS could contribute to improving GS of GM maize. Nevertheless, it is essential to establish a sufficient number of transects in GM and non-GM cultivation sites so that data can be used for effect detection (e.g. Lang *et al.*, 2019b).

Conclusions

Butterflies and their larval host plants were widespread and abundant in the maize agroecosystems surveyed and therefore they may be exposed to effects of GM maize cultivation. In contrast, few butterfly larvae were recorded in maize field margins and in most cases the sampling effort involved would be too high.

A number of adult butterfly indicators appeared suitable for GS. However, we found that the species most suitable for GS varied between maize-growing regions. For this reason, we strongly recommend to carry out a field survey in each differentiated receiving environment. The results of this study indicate that an improved GS could be implemented by monitoring selected butterfly indicators. However, further research is needed in order to determine which and how butterfly indicators should be monitored across wider areas where GM maizes are grown.

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Supplementary material Chapter 2

Appendix A. Composition of the multispecies butterfly indicators.

Table A.1. Multispecies butterfly indicators were constructed by aggregating species present in each site x year combination according to the following criteria: “migrant species” aggregated any of the 10 species that migrate across the study area (Stefanescu *et al.*, 2011b). “mobile species” aggregated any of the 12 species with high dispersion capacity in the study areas (Stefanescu *et al.*, 2011a) “low mobility species” aggregated any of the 18 species with low dispersion capacity in the study areas (Stefanescu *et al.*, 2011a).

Migrant species

Gegenes nostrodamus (Fabricius, 1793)
Lampides boeticus (L., 1767)
Leptotes pirithous (L., 1767)
Vanessa atalanta (L., 1758)
Vanessa cardui (L., 1758)
Papilio machaon L., 1758
Colias crocea (Geoffroy, 1785)
Pieris brassicae (L., 1758)
Pieris rapae (L., 1758)
Pontia daplidice (L., 1758)

Mobile species

Carcharodus alceae (Esper, 1780)
Celastrina argiolus (L., 1758)
Lycaena phlaeas (L., 1761)
Polyommatus icarus (Rottemburg, 1775)
Aglais io L., 1758
Aglais urticae (L., 1758)
Lasiommata megera (L., 1767)
Pararge aegeria (L., 1758)
Polygonia c-album (L., 1758)
Iphiclides feisthamelii (Duponchel, 1832)
Gonepteryx rhamni (L., 1758)
Pieris napi (L., 1758)

Low mobility or sedentary species

Carcharodus baeticus (Rambur, 1839)
Carcharodus flocciferus (Zeller, 1847)
Muschampia proto (Ochsenheimer, 1808)
Spialia sertorius (Hoffmannsegg, 1804)
Thymelicus lineola (Ochsenheimer, 1808)
Satyrium spini Dennis & Schiffermüller, 1775
Pyrgus malvoides (Elwes & Edwards, 1897)
Thymelicus acteon (Rottemburg, 1775)
Aricia agestis (Dennis & Schiffermüller, 1775)
Cupido argiades Pallas, 1771
Satyrium esculi (Hübner, 1804)
Coenonympha pamphilus (L., 1758)
Hipparchia fagi (Scopoli, 1763)
Maniola jurtina L., 1758
Melanargia lachesis Hübner, 1790
Pyronia bathseba (Fabricius, 1793)
Pyronia cecilia (Vallantin, 1894)
Pyronia tithonus (L., 1767)

Table A.2. Multispecies butterfly indicators were constructed by aggregating species present in each site x year combination according to the following criteria: "open habitat species" aggregated 16 species linked to open habitats in the area (from Herrando *et al.* 2016); finally, the "grassland indicators" aggregated 7 indicator species for European grasslands, an indicator developed by the European Environmental Agency (EEA, 2013).

Open habitat species	
Spialia sertorius (Hoffmannsegg, 1804)	*Pararge aegeria* (L., 1758)
Satyrium spini Dennis & Schiffermüller, 1775	*Aricia agestis* (Dennis & Schiffermüller, 1775)
Lampides boeticus (L., 1767)	*Satyrium esculi* (Hübner, 1804)
Vanessa atalanta (L., 1758)	*Coenonympha pamphilus* (L., 1758)
Papilio machaon (L., 1758)	*Hipparchia fagi* (Scopoli, 1763)
Pieris brassicae (L., 1758)	*Melanargia lachesis* Hübner, 1790
Celastrina argiolus (L., 1758)	*Pyronia tithonus* (L., 1767)
Polyommatus icarus (Rottemburg, 1775)	*Melitaea didyma* Esper, 1778
Grassland indicator species	
Coenonympha pamphilus (L., 1758)	*Polyommatus icarus* (Rottemburg, 1775)
Lasiommata megera (L., 1767)	*Spialia sertorius* (Hoffmannsegg, 1804)
Lycaena phlaeas (L., 1761)	*Thymelicus acteon* Rottemburg, 1775
Maniola jurtina L., 1758	

Appendix B. Butterfly species according to habitat.

Table B.1. Bujaraloz, butterfly species (Lepi doptera: Papilionoidea) recorded across different habitat types (alfalfa crop, maize field margin or noncrop area) in the maize-growing region of Bujaraloz, Aragón, Spain. Mean (m) number of butterflies per km and Standard deviation (SD) were calculated by averaging the number of butterflies recorded in 100 m transects in five sites. Butterflies were sampled in each site three times per growing season (2012 and 2013). The relative frequency (%Fr) is the percentage of the number of site x year combinations where the species was present. One protected species was detected (Near threatened (NT) according to the IUCN red list (Van Swaay *et al.*, 2010)).

			Alfalfa (N = 10)		**Margin (N = 10)**		**Noncrop (N = 9)**	
Family/group	**Species**	**IUCN**	**m ± SD**	**%Fr**	**m ± SD**	**%Fr**	**m ± SD**	**%Fr**
Hesperiidae	*Carcharodus alceae*		0.3 ± 1.1	10	0.7 ± 1.4	20	3.0 ± 5.1	33
	Carcharodus baeticus						0.4 ± 1.1	11
	Gegenes nostrodamus						0.4 ± 1.1	11
	Muschampia proto						0.7 ± 2.2	11
	Thymelicus acteon	NT	2.0 ± 5.3	20	3.0 ± 5.8	30	0.4 ± 1.1	11
Lycaenidae	*Lampides boeticus*		3.3 ± 6.3	40	1.7 ± 2.8	30		
	Leptotes pirithous		0.7 ± 1.4	20	0.7 ± 1.4	20		
	Polyommatus icarus		10.0 ± 8.5	90	16.3 ± 26.2	70	9.6 ± 11.4	78
Nymphalidae	*Lasiommata megera*		1.0 ± 2.2	20	1.0 ± 1.6	30	3.3 ± 5.8	33
	Pyronia cecilia		0.3 ± 1.1	10	0.7 ± 1.4	20	8.1 ± 11.1	67
	Vanessa cardui						0.4 ± 1.1	11
Pieridae	*Colias crocea*		13.3 ± 12.3	90	9.3 ± 12.	60	6.3 ± 5.9	78
	Pieris brassicae				0.3 ± 1.1	10	1.5 ± 3.4	22
	Pieris napi		9.7 ± 10.7	70	16.0 ± 17.2	80	7.4 ± 7.4	78
	Pieris rapae		27.0 ± 23.8	90	36.5 ± 30.0	100	23.3 ± 14.0	100
	Pontia daplidice		0.3 ± 1.1	10	1.0 ± 12.6	30	5.6 ± 6.7	56
Papilionoidea*	All		73.7 ± 48.2	100	94.7 ± 64.1	100	75.6 ± 36.1	100

*Papilionoidea also includes butterflies that were not identified to species.

Table B.2. Lleida butterfly species recorded across different habitats (alfalfa crop, maize field margin or noncrop area) in the maize-growing region of Lleida, Catalunya, Spain. Mean (m) number of butterflies per km and Standard deviation (SD) were calculated by averaging the number of butterflies recorded in 100 m transects in five sites. Butterflies were sampled in each site three times per growing season (2012 and 2013). The relative frequency (%Fr) is the percentage of the number of site x year combinations where the species was present.

			Alfalfa (N = 10)		**Margin (N = 10)**		**Noncrop (N = 9)**	
Family/group	**Species**	**IUCN**	**m ± SD**	**%Fr**	**m ± SD**	**%Fr**	**m ± SD**	**%Fr**
Hesperiidae	*Carcharodus alceae*		0.3 ± 1.1	10	0.8 ± 2.1	20	2.6 ± 4.3	44
	Spialia sertorius				0.3 ± 1.1	10		
Lycaenidae	*Lampides boeticus*		1.3 ± 3.2	20	0.3 ± 1.1	10	0.7 ± 1.5	22
	Leptotes pirithous				0.2 ± 0.5	10		
	Polyommatus icarus		12.3 ± 15.0	90	1.0 ± 1.6	30	17.0 ± 11.4	89
Nymphalidae	*Lasiommata megera*				0.3 ± 1.1	10	1.5 ± 2.9	22
	Pararge aegeria				0.3 ± 1.1	10		
	Pyronia cecilia						4.4 ± 7.3	33
	Vanessa cardui		0.3 ± 1.1	10	0.7 ± 1.4	20	4.8 ± 13.2	22
Papilionidae	*Papilio machaon*		0.3 ± 1.1	10	0.3 ± 1.1	10	3.3 ± 6.0	33
Pieridae	*Colias crocea*		3.7 ± 4.0	60	2.3 ± 2.2	60	5.6 ± 7.3	56
	Pieris brassicae		0.3 ± 1.1	10			1.1 ± 2.4	22
	Pieris napi		6.7 ± 11.4	50	12.0 ± 12.0	80	21.5 ± 26.6	78
	Pieris rapae		4.0 ± 5.4	60	5.0 ± 6.1	60	11.9 ± 15.9	78
	Pontia daplidice				0.3 ± 1.1	10	5.9 ± 6.0	78
Papilionoidea*	All		31.3 ± 21.6	100	26.7 ± 17.2	100	86.7 ± 62.3	100

*Papilionoidea also includes butterflies that were not identified to species.

Table B.3. (part 1) La Seu butterfly species (Hesperiidae, Lycaenidae and Nymphalidae) recorded across different habitats (alfalfa crop, maize field margin or noncrop area) in the maize-growing region of La Seu d'Urgell, Catalunya, Spain. Mean (m) number of butterflies per km and Standard deviation (SD) were calculated by averaging the number of butterflies recorded in 100 m transects in five sites. Butterflies were sampled in each site three times per growing season (2012 and 2013). The relative frequency (%Fr) is the percentage of the number of site x year combinations where the species was present. Three protected species were detected (Near threatened (NT) according to the IUCN red (Van Swaay *et al.*, 2010)).

			Alfalfa (N = 10)		**Margin (N = 10)**		**Noncrop (N = 10)**	
Family/group	**Species**	**IUCN**	**m ± SD**	**%Fr**	**m ± SD**	**%Fr**	**m ± SD**	**%Fr**
Hesperiidae	*Carcharodus alceae*		0.7 ± 1.4	20	0.3 ± 1.1	10	0.3 ± 1.1	10
	Carcharodus flocciferus	NT					0.7 ± 2.1	10
	Pyrgus malvoides		0.3 ± 1.1	10	0.3 ± 1.1	10	0.3 ± 1.1	10
	Thymelicus acteon	NT					0.3 ± 1.1	10
	Thymelicus lineola						0.3 ± 1.1	10
Lycaenidae	*Aricia agestis*				0.3 ± 1.1	10		
	Celastrina argiolus				0.3 ± 1.1	10		
	Cupido argiades						0.7 ± 1.4	20
	Lampides boeticus		39.7 ± 94.1	90	3.3 ± 4.7	50	2.0 ± 3.6	30
	Leptotes pirithous		0.7 ± 1.4	20			0.3 ± 1.1	10
	Lycaena phlaeas		0.3 ± 1.1	10	0.3 ± 1.1	10	0.3 ± 1.1	10
	Polyommatus icarus		10.0 ± 19.5	60	2.7 ± 5.2	40	1.7 ± 3.2	30
	Satyrium esculi						1.0 ± 3.2	10
	Satyrium spini		0.3 ± 1.1	10				
Nymphalidae	*Aglais io*		2.0 ± 4.5	20	1.3 ± 1.7	40	1.0 ± 3.2	10
	Aglais urticae						0.7 ± 1.4	20
	Coenonympha pamphilus		0.3 ± 1.1	10	0.7 ± 2.1	10	1.0 ± 2.2	20
	Hipparchia fagi	NT	0.3 ± 1.1	10	0.3 ± 1.1	10	2.3 ± 4.5	30
	Lasiommata megera		1.0 ± 1.6	30	1.7 ± 2.4	40	2.0 ± 2.8	40
	Maniola jurtina		0.3 ± 1.1	10	0.7 ± 1.4	20	1.0 ± 2.2	20
	Melanargia lachesis				0.7 ± 1.4	20	1.7 ± 2.4	40
	Melitaea didyma						0.3 ± 1.1	10
	Pararge aegeria				1.0 ± 2.2	20	4.7 ± 6.3	50
	Polygonia c-album		0.3 ± 1.1	10			0.7 ± 1.4	20
	Pyronia bathseba						0.3 ± 1.1	10
	Pyronia cecilia						3.3 ± 6.3	30
	Pyronia tithonus		0.7 ± 1.4	20			1.7 ± 2.8	30
	Vanessa atalanta				0.3 ± 1.1	10		
	Vanessa cardui		0.7 ± 1.4	20	1.3 ± 2.3	30	0.7 ± 2.1	10

Table B.3. (part 2) Butterfly species (Papilionidae, Pieridae and the group of all Papilionoidea specimens) recorded across different habitats (alfalfa crop, maize field margin or noncrop area) in the maize-growing region of La Seu d'Urgell, Spain. Mean (m) number of butterflies per km and Standard deviation (SD) were calculated by averaging the number of butterflies recorded in 100 m transects in five sites. Butterflies were sampled in each site three times per growing season (2012 and 2013). The relative frequency (%Fr) is the percentage of the number of site x year combinations where the species was present. Three protected species were detected (Near threatened (NT) according to the IUCN red list red (Van Swaay *et al.*, 2010).

			Alfalfa (N = 10)		**Margin (N = 10)**		**Noncrop (N = 10)**	
Family	**Taxonomy**	**IUCN**	**m ± SD**	**%Fr**	**m ± SD**	**%Fr**	**m ± SD**	**%Fr**
Papilionidae	*Iphiclides feisthamelii*		0.3 ± 1.1	10			0.3 ± 1.1	10
	Papilio machaon		0.3 ± 1.1	10	0.3 ± 1.1	10		
Pieridae	*Colias crocea*		6.0 ± 2.1	100	3.3 ± 2.7	80	3.0 ± 5.3	40
	Gonepteryx rhamni						0.3 ± 1.1	10
	Pieris brassicae		3.0 ± 5.8	30			3.3 ± 3.8	50
	Pieris napi		4.3 ± 5.7	80	3.7 ± 3.3	70	8.7 ± 7.1	80
	Pieris rapae		3.7 ± 5.8	40	6.0 ± 6.4	70	13.3 ± 13.7	80
	Pontia daplidice		0.3 ± 1.1	10			0.7 ± 1.4	20
Papilionoidea	All		87.0 ± 114.0	100	32.0 ± 15.1	100	64.3 ± 32.9	100

*Papilionoidea also includes butterflies that were not identified to species.

Appendix C. Larval host plant distribution.

Table C.1.1. Host plants of butterfly species (Hesperiidae and Lycaenidae) in maize agroecosystems in Bujaraloz. The table shows results from: a) survey of maize weeds (Juárez-Escario *et al.*, 2018); b) literature search (Pujol i Palol, 2017; MAPA, 2020) on crops used as larval host plants; and c) flora relevés from maize field margins and noncrop vegetation in five sites (percent cover and standard deviation shown for 30 m^2), occurrence of the larval host plants (%Fr) is proportion of sites.

Butterfly		**Larval host plant**			**Maize weeds**	**Crop**	**Margin**			**Noncrop**		
Family	**species**	**Family**	**Genus**	**species**			**M**	**SD**	**%Fr**	**M**	**SD**	**%Fr**
Hesperiidae	*Carcharodus alceae*	Malvaceae	*Malva*	*sylvestris*	x		1.7	2.8	60			
				neglecta	x		0.1	0.0	20			
			Abutilon	*theophrasti*	x		5.0	0.0	20			
	Carcharodus baeticus	Lamiaceae										
	Gegenes nostrodamus	Poaceae	*Zea*	*mays*		x						
			Panicum	*sp.*	x							
	Muschampia proto	Lamiaceae	*Phlomis*	*lychnitis*						5.0	0.0	20
	Thymelicus acteon	Poaceae	*Dactylis*	*glomerata*		x				1.7	2.8	60
Lycaenidae	*Lampides boeticus*	Asteraceae	*Aster*	*squamatus*	x		2.6	2.8	80	0.1	0.0	20
			Conyza	*sumatrensis*	x							
				sp.	x		7.5	9.0	60			
		Fabaceae	*Genista*	*sp.*						5.0	0.0	40
			Lotus	*corniculatus*			5.0	0.0	20			
			Medicago	*sativa*	x	x	3.8	2.5	80	5.0	0.0	20
			Phaseolus	*vulgaris*		x						
			Pisum	*sativum*		x						
		Plantaginaceae	*Plantago*	*coronopus*			0.1	0.0	20			
	Leptotes pirithous	Lamiaceae	*Salvia*	*rosmarinus*						5.0	0.0	20
		Lythraceae	*Lythrum*	*salicaria*								
		Fabaceae	*Lotus*	*corniculatus*		x	5.0	0.0	20			
			Medicago	*sativa*	x	x	3.8	2.5	80	5.0	0.0	20
		Apiaceae	*Pastinaca*	*sativa*		x						
	Polyommatus icarus	Fabaceae	*Lotus*	*corniculatus*		x	5.0	0.0	20			
			Medicago	*sativa*	x	x	3.8	2.5	80	5.0	0.0	20
			Trifolium	*pratense*		x						
				repens		x	0.1	0.0	20			

Table C.1.2. Host plants of butterfly species (Nymphalidae and Pieridae) in maize agroecosystems in Bujaraloz. The table shows the results from: a) a survey of maize weeds (Juárez-Escario *et al.*, 2018); b) a literature search (Pujol i Palol, 2017; MAPA, 2020) on crops used as larval host plants; and c) flora relevés conducted in maize field margins and non-crop vegetation in five sites (mean percent cover and standard deviation is shown for 30 m2), the occurrence of the larval host plants (%Fr) is shown as the proportion of sites where it was present.

Butterfly		**Larval host plant**			**Maize weeds**	**Crop**	**Margin**			**Noncrop**		
Family	**species**	**Family**	**Genus**	**species**			**M**	**SD**	**%Fr**	**M**	**SD**	**%Fr**
Nymphalidae	*Lasiommata megera*	Poaceae	*Cynodon*	*dactylon*	x		18.8	26.4	40	0.1	0.0	20
	Pyronia cecilia	Poaceae	*Brachypodium*	*retusum*						0.1	0.0	40
	Vanessa cardui	Asteraceae	*Carduus*	*sp.*			2.6	3.5	40	5.0	0.0	20
			Centaurea	*sp.*			2.6	3.5	40	15.9	18.8	60
			Cirsium	*arvense*	x							
				sp.			5.0	0.0	20			
			Cynara	*cardunculus*		x						
			Picris	*echioides*	x		3.4	2.8	60			
		Malvaceae	*Malva*	*neglecta*	x		0.1	0.0	20			
				sylvestris	x		1.7	2.8	60			
		Plantaginaceae	*Plantago*	*lanceolata*			1.7	2.8	60			
Pieridae	*Colias crocea*	Fabaceae	*Lotus*	*corniculatus*		x	5.0	0.0	20			
			Medicago	*sativa*	x	x	3.8	2.5	80	5.0	0.0	20
			Trifolium	*pratense*		x						
				repens		x	0.1	0.0	20			
	Pieris brassicae	Brassicaceae	*Brassica*	*oleracea*		x						
				napus		x						
			Diplotaxis	*erucoides*	x							
			Lepidium	*draba*			0.1	0.0	20			
	Pieris napi	Brassicaceae	*Cardamine*	*hirsuta*	x							
			Lepidium	*draba*			0.1	0.0	20			
	Pieris rapae	Brassicaceae	*Brassica*	*oleracea*		x						
			Capsella	*bursa-pastoris*	x							
			Diplotaxis	*erucoides*	x							
			Lepidium	*draba*			0.1	0.0	20			
	Pontia daplidice	Brassicaceae	*Diplotaxis*	*erucoides*	x	x						
			Lepidium	*draba*			0.1	0.0	20			

Table C.2.1. Host plants of butterfly species (Hesperiidae and Lycaenidae) in maize agroecosystems in Lleida. The table shows the results from: a) a survey of maize weeds (Juárez-Escario *et al.*, 2018); b) a literature search (Pujol i Palol, 2017; MAPA, 2020) on crops used as larval host plants; and c) flora relevés conducted in maize field margins (22 sites) and non-crop vegetation (5 sites) (mean percent cover and standard deviation is shown for 100 m^2 and 30 m^2 respectively), the occurrence of the larval host plants (%Fr) is shown as the proportion of sites where it was present.

Butterfly		Larval host plant			Maize weeds	Crop	Margin*			Noncrop		
Family	**species**	**Family**	**Genus**	**species**			**M**	**SD**	**%Fr**	**M**	**SD**	**%Fr**
Hesperiidae	*Carcharodus alceae*	Malvaceae	*Malva*	*sylvestris*	x		1.7	1.7	91	0.1	0.0	20
				neglecta	x		0.1	0.0	5			
			Abutilon	*theophrasti*	x		0.4	0.5	45			
	Spialia sertorius	Rosaceae	*Sanguisorba*	*minor*			0.0	0.0	5			
Lycaenidae	*Lampides boeticus*	Asteraceae	*Aster*	*squamatus*	x		0.2	0.2	55			
			Conyza	*sumatrensis*	x							
				sp.	x		0.7	0.5	55	0.1	0.0	20
		Fabaceae	*Astragalus*	*sp.*						0.1	0.0	20
			Genista	*sp.*						8.8	12.3	40
			Lotus	*corniculatus*		x						
			Medicago	*sativa*	x	x	3.9	6.2	64	0.1	0.0	40
			Onobrychis	*sp.*		x						
			Phaseolus	*vulgaris*		x						
			Pisum	*sativum*		x						
			Trifolium	*pratense*		x	0.2	0.1	14	0.1	0.0	20
		Plantaginaceae	*Plantago*	*coronopus*			0.5	0.7	14			
	Leptotes pirithous	Fabaceae	*Astragalus*	*sp.*						0.1	0.0	20
			Lotus	*corniculatus*		x						
			Medicago	*sativa*	x	x	3.9	6.2	64	0.1	0.0	40
			Trifolium	*pratense*		x	0.2	0.1	14	0.1	0.0	20
		Apiaceae	*Pastinaca*	*sativa*		x						
	Polyommatus icarus	Fabaceae	*Dorycnium*	*pentaphyllum*			4.1	3.2	14	2.6	3.5	40
			Lotus	*corniculatus*		x						
			Medicago	*lupulina*			0.1	0.0	9			
				sativa	x	x	3.9	6.2	64	0.1	0.0	40
			Ononis	*spinosa*			2.4	2.3	23	0.1	0.0	40
			Trifolium	*arvense*								
				pratense		x	0.2	0.1	14	0.1	0.0	20
				repens		x	0.4	0.1	9	0.1	0.0	20

*In Lleida field margins were sampled more intensively than in the other regions.

Table C.2.2. Host plants of butterfly species (Nymphalidae and Papilionidae) in maize agroecosystems in Lleida. The table shows the results from: a) a survey of maize weeds (Juárez-Escario *et al.*, 2018); b) a literature search (Pujol i Palol, 2017; MAPA, 2020) on crops used as larval host plants; and c) flora relevés conducted in maize field margins (22 sites) and non-crop vegetation (5 sites) (mean percent cover and standard deviation is shown for 100 m^2 and 30 m^2 respectively), the occurrence of the larval host plants (%Fr) is shown as the proportion of sites where it was present.

Butterfly		**Larval host plant**			**Maize weeds**	**Crop**	**Margin***			**Noncrop**		
Family	**species**	**Family**	**Genus**	**species**			**M**	**SD**	**%Fr**	**M**	**SD**	**%Fr**
Nymphalidae	*Lasiommata megera*	Poaceae	*Cynodon*	*dactylon*	x		1.5	1.6	95	17.5	0.0	20
	Pararge aegeria	Poaceae	*Elymus*	*sp.*			1.6	2.7	41	2.6	3.5	40
			Dactylis	*glomerata*		x				0.1	0.0	40
			Poa	*sp.*	x		0.1	0.1	9			
	Pyronia cecilia	Poaceae	*Brachypodium*	*retusum*						62.5	35.4	40
	Vanessa cardui	Asteraceae	*Carduus*	*sp.*			0.4	0.8	23	0.1	0.0	20
			Centaurea	*sp.*						0.2	0.1	40
			Cirsium	*arvense*	x							
				sp.			1.2	1.7	23			
			Cynara	*cardunculus*		x						
			Picris	*echioides*	x		1.2	2.0	18	0.1	0.0	40
				sp.	x		2.0	3.8	64			
			Silybum	*marianum*			0.4	0.6	9			
		Malvaceae	*Malva*	*neglecta*	x		0.1		5			
				sylvestris	x		1.7	1.7	91	0.1	0.0	20
		Plantaginaceae	*Plantago*	*lanceolata*			1.1	1.8	18	0.1	0.0	20
Papilionidae	*Papilio machaon*	Apiaceae	*Foeniculum*	*vulgare*			2.5	3.5	64	2.6	3.5	40
			Pastinaca	*sativa*		x						

*In Lleida, field margins were sampled more intensively than in the other regions as part of a directed larval survey.

Table C.2.3. Host plants of butterfly species (Pieridae) in maize agroecosystems in Lleida. The table shows the results from: a) a survey of maize weeds (Juárez-Escario *et al.*, 2018); b) a literature search (Pujol i Palol, 2017; MAPA, 2020) on crops used as larval host plants; and c) flora relevés conducted in maize field margins (22 sites) and non-crop vegetation (5 sites) (mean percent cover and standard deviation is shown for 100 m^2 and 30 m^2 respectively), the occurrence of the larval host plants (%Fr) is shown as the proportion of sites where it was present.

Butterfly		**Larval host plant**			**Maize weeds**	**Crop**	**Margin***			**Noncrop**		
Family	**species**	**Family**	**Genus**	**species**			**M**	**SD**	**%Fr**	**M**	**SD**	**%Fr**
Pieridae	*Colias crocea*	Fabaceae	*Astragalus*	*sp*						0.1	0.0	20
			Lotus	*corniculatus*		x						
			Medicago	*lupulina*			0.1	0.0	9			
				sativa	x	x	3.9	6.2	64	0.1	0.0	40
			Melilotus	*officinalis*			1.4		5	0.1	0.0	20
			Trifolium	*pratense*		x	0.2	0.1	14	0.1	0.0	20
				repens		x	0.4	0.1	9	0.1	0.0	20
	Pieris brassicae	Brassicaceae	*Brassica*	*oleracea*		x						
				napus		x						
			Diplotaxis	*erucoides*	x		0.1	0.2	27	0.1	0.0	20
			Lepidium	*draba*			0.4	0.5	18			
			Sisymbrium	*sp.*			0.3	0.0	5			
	Pieris napi	Brassicaceae	*Cardamine*	*hirsuta*	x							
			Lepidium	*draba*			0.4	0.5	18			
	Pieris rapae	Brassicaceae	*Brassica*	*oleracea*		x						
			Capsella	*bursa-pastoris*	x		0.1	0.1	18			
			Diplotaxis	*erucoides*	x		0.1	0.2	27	0.1	0.00	20
			Lepidium	*draba*			0.4	0.5	18			
	Pontia daplidice	Brassicaceae	*Diplotaxis*	*erucoides*	x		0.1	0.2	27	0.1	0.00	20
			Lepidium	*draba*			0.4	0.5	18			

*In Lleida field margins were sampled more intensively than in the other regions.

Table C.3.1. Host plants of butterfly species (Hesperiidae and Papilionidae) in maize agroecosystems in La Seu. The table shows the results from: a) a survey of maize weeds (Juárez-Escario *et al.*, 2018); b) a literature search (Pujol i Palol, 2017; MAPA, 2020) on crops used as larval host plants; and c) flora relevés conducted in maize field margins and non-crop vegetation in five sites (mean percent cover and standard deviation is shown for 30 m2), the occurrence of the larval host plants (%Fr) is shown as the proportion of sites where it was present.

Butterfly		**Larval host plant**			**Maize weeds**	**Crop**	**Margin**			**Noncrop**		
Family	**species**	**Family**	**Genus**	**species**			**M**	**SD**	**%Fr**	**M**	**SD**	**%Fr**
Hesperiidae	*Carcharodus alceae*	Malvaceae	*Malva*	*sylvestris*	x		0.1	0.0	20			
				neglecta	x							
			Abutilon	*theophrasti*	x		0.1	0.0	20			
	Carcharodus flocciferus	Lamiaceae										
	Pyrgus malvoides	Rosaceae	*Potentilla*	*reptans*						0.1	0.0	20
			Rubus	*ulmifolius*						5.0	0.0	60
	Thymelicus acteon	Poaceae	*Dactylis*	*glomerata*		x				37.5	0.0	20
	Thymelicus lineola	Poaceae										
Papilionidae	*Iphiclides feisthamelii*	Rosaceae	*Prunus*	*spinosa*						2.6	3.5	40
				domestica		x						
				dulcis		x	5.0		20	5.0	0.0	20
				avium		x						
			Pyrus	*communis*		x						
			Crataegus	*monogyna*						2.6	3.5	40
	Papilio machaon	Apiaceae	*Foeniculum*	*vulgare*			2.6	3.5	40			
			Daucus	*carota*						5.0	0.0	20
			Pastinaca	*sativa*		x						

Table C.3.2. Host plants of butterfly species (Lycaenidae, part 1) in maize agroecosystems in La Seu. The table shows the results from: a) a survey of maize weeds (Juárez-Escario *et al.*, 2018); b) a literature search (Pujol i Palol, 2017; MAPA, 2020) on crops used as larval host plants; and c) flora relevés conducted in maize field margins and non-crop vegetation in five sites (mean percent cover and standard deviation is shown for 30 m2), the occurrence of the larval host plants (%Fr) is shown as the proportion of sites where it was present.

Butterfly		**Larval host plant**			**Maize weeds**	**Crop**	**Margin**			**Noncrop**		
Family	**species**	**Family**	**Genus**	**species**			**M**	**SD**	**%Fr**	**M**	**SD**	**%Fr**
Lycaenidae (part 1)	*Aricia agestis*	Cistaceae										
	Celastrina argiolus	Araliaceae	*Hedera*	*helix*						0.1	0.0	20
		Cannabaceae	*Humulus*	*lupulus*		x				5.0	0.0	20
		Fabaceae	*Medicago*	*sativa*	x	x	3.8	2.5	80			
			Vicia	*cracca*						0.1	0.0	20
		Rosaceae	*Rubus*	*ulmifolius*						5.0	0.0	60
				sp.	x		1.7	2.8	60	5.0	0.0	80
	Cupido argiades	Fabaceae	*Trifolium*	*pratense*		x	0.1	0.0	20			
			Medicago	*sativa*	x	x	3.8	2.5	80			
			Lotus	*corniculatus*		x				0.1	0.0	40
	Lampides boeticus	Asteraceae	*Aster*	*squamatus*	x		0.1	0.0	20			
			Conyza	*sumatrensis*	x							
				sp.	x		0.1	0.0	20			
		Fabaceae	*Lotus*	*corniculatus*		x				0.1	0.0	40
			Hedysarum	*sp.*		x						
			Medicago	*sativa*	x	x	3.8	2.5	80			
			Onobrychis	*sp.*		x						
			Phaseolus	*vulgaris*		x						
			Pisum	*sativum*		x						
			Trifolium	*pratense*		x	0.1	0.0	20			

Table C.3.3. Host plants of butterfly species (Lycaenidae, continued from previous table) in maize agroecosystems in La Seu. The table shows the results from: a) a survey of maize weeds (Juárez-Escario *et al.*, 2018); b) a literature search (Pujol i Palol, 2017; MAPA, 2020) on crops used as larval host plants; and c) flora relevés conducted in maize field margins and non-crop vegetation in five sites (mean percent cover and standard deviation is shown for 30 m2), the occurrence of the larval host plants (%Fr) is shown as the proportion of sites where it was present.

Butterfly		**Larval host plant**			**Maize weeds**	**Crop**	**Margin**			**Noncrop**		
Family	**species**	**Family**	**Genus**	**species**			**M**	**SD**	**%Fr**	**M**	**SD**	**%Fr**
Lycaenidae (part 2)	*Leptotes pirithous*	Lythraceae	*Lythrum*	*salicaria*			0.1	0.0	20			
		Fabaceae	*Lotus*	*corniculatus*		x				0.1	0.0	40
			Medicago	*sativa*	x	x	3.8	2.5	80			
			Trifolium	*pratense*		x	0.1	0.0	20			
		Apiaceae	*Pastinaca*	*sativa*		x						
	Lycaena phlaeas	Polygonaceae	*Rumex*	*crispus*	x							
				sp.	x							
	Polyommatus icarus	Fabaceae	*Lotus*	*corniculatus*		x				0.1	0.0	40
			Medicago	*sativa*	x	x	3.8	2.5	80			
			Trifolium	*pratense*		x	0.1	0.0	20			
				repens		x	5.0	0.0	20			
	Satyrium esculi	Fagaceae	*Quercus*	*coccifera*						5.0	0.0	20
				ilex						37.5	0.0	20
				pyrenaica						5.0	0.0	20
	Satyrium spini	Rhamnaceae										

Table C.3.4. Host plants of butterfly species (Nymphalidae, part 1) in maize agroecosystems in La Seu. The table shows the results from: a) a survey of maize weeds ; b) a literature search (Pujol i Palol, 2017; MAPA, 2020) on crops used as larval host plants; and c) flora relevés conducted in maize field margins and non-crop vegetation in five sites (mean percent cover and standard deviation is shown for 30 m2), the occurrence of the larval host plants (%Fr) is shown as the proportion of sites where it was present.

Butterfly		**Larval host plant**			**Maize weeds**	**Crop**	**Margin**			**Noncrop**		
Family	**species**	**Family**	**Genus**	**species**			**M**	**SD**	**%Fr**	**M**	**SD**	**%Fr**
Nymphalidae (part 1)	*Aglais io*	Urticaceae	*Urtica*	*dioica*						8.8	12.3	40
		Cannabaceae	*Humulus*	*lupulus*		x						
	Aglais urticae	Urticaceae	*Urtica*	*dioica*						8.8	12.3	40
	Coenonympha pamphilus	Poaceae										
	Hipparchia fagi	Poaceae	*Festuca*	*sp.*	x	x						
	Lasiommata megera	Poaceae	*Cynodon*	*dactylon*	x		11.7	10.0	60			
	Maniola jurtina	Poaceae	*Elymus*	*sp.*			5.0		20	12.6	21.6	60
			Poa	*annua*	x							
			Cynodon	*dactylon*	x		11.7	10.0	60			
	Melanargia lachesis	Poaceae	*Bromus*	*sp.*	x	x				0.1	0.0	20
			Dactylis	*glomerata*		x				37.5	0.0	20
			Elymus	*sp.*			5.0		20	12.6	21.6	60
	Melitaea didyma	Plantaginaceae	*Plantago*	*lanceolata*			5.0		20	0.1	0.0	20
				sp.	x		5.0		20	0.1	0.0	20
	Pararge aegeria	Poaceae	*Elymus*	*sp.*			5.0		20	12.6	21.6	60
			Dactylis	*glomerata*		x				37.5	0.0	20
	Polygonia c-album	Urticaceae	*Urtica*	*dioica*						8.8	12.3	40
		Cannabaceae	*Humulus*	*lupulus*		x						
		Ulmaceae	*Celtis*	*australis*			5.0		20	21.3	23.0	40

Table C.3.5. Host plants of butterfly species (Nymphalidae, continued from previous table) in maize agroecosystems in La Seu. The table shows the results from: a) a survey of maize weeds (Juárez-Escario *et al.*, 2018); b) a literature search (Pujol i Palol, 2017; MAPA, 2020) on crops used as larval host plants; and c) flora relevés conducted in maize field margins and non-crop vegetation in five sites (mean percent cover and standard deviation is shown for 30 m2), the occurrence of the larval host plants (%Fr) is shown as the proportion of sites where it was present.

Butterfly		**Larval host plant**			**Maize weeds**	**Crop**	**Margin**			**Noncrop**		
Family	**species**	**Family**	**Genus**	**species**			**M**	**SD**	**%Fr**	**M**	**SD**	**%Fr**
Nymphalidae (part 2)	*Pyronia bathseba*	Poaceae										
	Pyronia cecilia	Poaceae										
	Pyronia tithonus	Poaceae	*Poa*	*annua*	x							
				sp.	x	x						
	Vanessa atalanta	Urticaceae	*Urtica*	*dioica*						8.8	12.3	40
	Vanessa cardui	Asteraceae	*Arctium*	*sp.*			0.1	0.0	20	0.1	0.0	20
			Carduus	*sp.*			0.1	0.0	40	0.1	0.0	20
			Centaurea	*sp.*						0.1	0.0	20
		Asteraceae	*Cirsium*	*arvense*	x							
			Picris	*echioides*	x							
			Silybum	*marianum*								
		Malvaceae	*Malva*	*neglecta*	x							
				sylvestris	x		0.1	0.0	20			
		Plantaginaceae	*Plantago*	*lanceolata*			5.0	0.0	20	0.1	0.0	20
		Urticaceae	*Urtica*	*dioica*						8.8	12.3	40

Table C.3.6. Host plants of butterfly species (Pieridae) in maize agroecosystems in La Seu. The table shows the results from: a) a survey of maize weeds (Juárez-Escario *et al.*, 2018); b) a literature search (Pujol i Palol, 2017; MAPA, 2020) on crops used as larval host plants; and c) flora relevés conducted in maize field margins and non-crop vegetation in five sites (mean percent cover and standard deviation is shown for 30 m2), the occurrence of the larval host plants (%Fr) is shown as the proportion of sites where it was present.

Butterfly		**Larval host plant**			**Maize weeds**	**Crop**	**Margin**			**Noncrop**		
Family	**species**	**Family**	**Genus**	**species**			**M**	**SD**	**%Fr**	**M**	**SD**	**%Fr**
Pieridae	*Colias crocea*	Fabaceae	*Lotus*	*corniculatus*		x				0.1	0.0	40
			Medicago	*sativa*	x	x	3.8	2.5	80			
			Melilotus	*officinalis*			5.0	0.0	20	2.6	3.5	40
			Trifolium	*pratense*		x	0.1	0.0	20			
				repens		x	5.0	0.0	20			
			Vicia	*cracca*						0.1	0.0	20
	Gonepteryx rhamni	Rhamnaceae										
	Pieris brassicae	Brassicaceae	*Brassica*	*oleracea*		x						
				napus		x						
			Diplotaxis	*erucoides*	x							
	Pieris napi	Brassicaceae	*Cardamine*	*hirsuta*	x							
	Pieris rapae	Brassicaceae	*Brassica*	*oleracea*		x						
			Capsella	*bursa-pastoris*	x							
			Diplotaxis	*erucoides*	x							
	Pontia daplidice	Brassicaceae	*Diplotaxis*	*erucoides*	x							

Appendix D. Butterfly indicator selection process.

Table D.1.1. Exclusion criteria, butterflies were excluded from the candidate list if they were not exposed or not susceptible to *Bt* toxins or herbicide, or incapable of reflecting changes due to GM maize cultivation. In grey, the species excluded from the selection process.

	STEP A.1 Exposure			STEP A.2 Susceptibility			STEP A.3. Responsiveness			
Species	Larval feeding habit	Larval overlap with pollen shed	**Exposed**	Susceptible to *Bt* toxins	Susceptible to herbicide	**Susceptible**	Mobility	Pest	**Responsive**	**EXCLUDE**
Aglais io	surface feeder	yes	**yes**	very	nd	**yes**	mobile		**yes**	**NO**
Carcharodus alceae	surface feeder	yes	**yes**	nd	nd		mobile		**yes**	**NO**
Coenonympha pamphilus	surface feeder	yes	**yes**	nd	nd		low mob		**yes**	**NO**
Cupido argiades	surface feeder	yes	**yes**	nd	nd		low mob		**yes**	**NO**
Lasiommata megera	surface feeder	yes	**yes**	nd	nd		mobile		**yes**	**NO**
Lycaena phlaeas	surface feeder	yes	**yes**	nd	nd		mobile		**yes**	**NO**
Melanargia lachesis	surface feeder	yes, 1 ann. gen	**yes**	nd	nd		low mob		**yes**	**NO**
Pararge aegeria	surface feeder	yes	**yes**	nd	nd		mobile		**yes**	**NO**
Pieris napi	surface feeder	yes	**yes**	moderate	nd	**yes**	mobile		**yes**	**NO**
Polyommatus icarus	surface feeder	yes	**yes**	nd	nd		mobile		**yes**	**NO**
Pyrgus malvoides	surface feeder	yes	**yes**	nd	nd		low mob		**yes**	**NO**
Pyronia cecilia	surface feeder	yes, 1 ann. gen	**yes**	nd	nd		low mob		**yes**	**NO**
Pyronia tithonus	surface feeder	yes, 1 ann. gen	**yes**	nd	nd		low mob		**yes**	**NO**
Thymelicus acteon	surface feeder	yes, 1 ann. gen	**yes**	nd	nd		low mob		**yes**	**NO**
Colias crocea	surface feeder	yes	**yes**	nd	nd		migrant		**no**	**YES**
Lampides boeticus	Endophytic	yes	**no**	nd	nd		migrant		**no**	**YES**
Leptotes pirithous	endophytic til L3	yes	**yes**	nd	nd		migrant		**no**	**YES**
Papilio machaon	surface feeder	yes	**yes**	yes	nd	**yes**	migrant		**no**	**YES**
Pieris brassicae	surface feeder	yes	**yes**	yes	nd		migrant	yes	**no**	**YES**
Pieris rapae	surface feeder	yes	**yes**	yes	nd		migrant	yes	**no**	**YES**
Pontia daplidice	surface feeder	yes	**yes**	nd	nd		migrant		**no**	**YES**
Vanessa cardui	surface feeder	yes	**yes**	nd	nd		migrant		**no**	**YES**

Table D.2.1. Prioritisation criteria applied to butterfly species across the entire study area, to select the candidates most appropriate for monitoring possible impacts of *Bt* or GMHT maize on butterflies species were prioritised according to the highest exposure in the field, susceptibility to *Bt* or herbicide, species that could be expected to be most responsive to changes, protected species and species with most information regarding ecology and biology.

	STEP B.1 Exposure		**STEP B.2 Susceptibility to *Bt* toxin/Herbicide**		**STEP B.3 Responsiveness**				**STEP B.4 Conservation value**		**STEP B.5 Information ecology and biology**				
	Larva feeds on maize	**Larval host plant near maize**	**Herbicide**	***Bt***	**Mobility**		**Habitat preference**		**IUCN**		**Availability information (refs WOK)**		**Indicator**		
Species	**score**	**score**	**score**	**Score**	**Type**	**score**	**Type**	**score**	**category**	**Score**	**N refs**	**score**	**Type**	**score**	**FINAL RANK**
Thymelicus acteon		1			low mob	0.5	open	0.5	NT	1	8	0.06	Gind	1	4.1
Aglais io		1		1	mobile		closed		LC		107	0.83	*Bt*	1	3.8
Coenonympha pamphilus		1			low mob	0.5	open	0.5	LC		50	0.39	Gind	1	3.4
Carcharodus flocciferus†					metapop	1.0			NT	1	1	0.01			2.0
Lycaena phlaeas		1			mobile		open	0.5	LC		26	0.20	Gind	1	2.7
Polyommatus icarus		1			mobile		open	0.5	LC		18	0.14	Gind	1	2.6
Hipparchia fagi†		1			low mob	0.5			NT	1	3	0.02			2.5
Pieris napi		1		1	mobile		closed		LC		61	0.47			2.5
Pyronia tithonus		1			low mob	0.5	open	0.5	LC		34	0.26			2.3
Melanargia lachesis		1			low mob	0.5	open	0.5	LC		6	0.05			2.0
Lasiommata megera		1			mobile				LC		5	0.04	Gind	1	2.0
Cupido argiades		1			low mob	0.5	open	0.5	LC		3	0.02			2.0
Pyrgus malvoides		1			low mob	0.5	open	0.5	LC		1	0.01			2.0
Pyronia cecilia		1			low mob	0.5	open	0.5	LC		1	0.01			2.0
Gegenes nostrodamus†	1	1			migrant				LC			0.00			2.0
Pararge aegeria		1			mobile		closed		LC		38	0.29			1.3
Carcharodus alceae		1			mobile				LC		1	0.01			1.0

†Species that were not frequent but that were of special interest as indicators (maize-feeding species or high conservation value).

Table D.2.2. Prioritisation criteria applied to butterfly species in Bujaraloz, to select the candidates most appropriate for monitoring possible impacts of *Bt* or GMHT or maize on butterflies we prioritised species according to the highest exposure in the field, susceptibility to *Bt* or herbicide, species that could be expected to be most responsive to changes, protected species and species with most information regarding ecology and biology.

	STEP B.1 Exposure			STEP B.2 Susceptibility to *Bt* toxin/Herbicide		STEP B.3 Responsiveness				STEP B.4 Conservation value		STEP B.5 Information ecology and biology				
	Adults present both years	Larva feeds on maize	Larval host plant near maize	Herbicide	*Bt*	Mobility		Habitat preference		IUCN		Availability information (refs WOK)		Indicator		
Species	**score**	**score**	**score**	**score**	**Score**	**Type**	**score**	**Type**	**score**	**category**	**Score**	**N refs**	**score**	**Type**	**score**	**RANK Bujaraloz**
Thymelicus acteon	1		1			low mob	0.5	open	0.5	NT	1	21	0.0	Gind	1	5.0
Pieris napi	1		1		1	mobile		closed		LC		428	1.0			4.0
Polyommatus icarus	1		1			mobile		open	0.5	LC		150	0.4	Gind	1	3.9
Lasiommata megera	1		1			mobile				LC		48	0.1	Gind	1	3.1
Pyronia cecilia	1		1			low mob	0.5	open	0.5	LC		10	0.0			3.0
Carcharodus alceae	1		1			mobile				LC		14	0.0			2.0
Gegenes nostrodamus		1				migrant				LC		7	0.0			1.0

Table D.2.3. Prioritisation criteria applied to species in Lleida, to select the candidates most appropriate for monitoring possible impacts of *Bt* or GMHT or maize on butterflies we prioritised species according to the highest exposure in the field, sensitivity to *Bt* or herbicide, species that could be expected to be most responsive to changes, protected species and species with most information regarding ecology and biology.

	STEP B.1 Exposure			**STEP B.2 Susceptibility to *Bt* toxin/Herbicide**		**STEP B.3 Responsiveness**				**STEP B.4 Conservation value**		**STEP B.5 Information ecology and biology**				
	Adults present both years	**Larva found at anthesis**	**Larval host plant near maize**	**Herbicide**	***Bt***	**Mobility**		**Habitat preference**		**IUCN**		**Availability information (refs WOK)**		**Indicator**		
Species	**score**	**score**	**score**	**score**	**Score**	**Type**	**score**	**Type**	**score**	**category**	**Score**	**Nrefs**	**score**	**Type**	**score**	**RANK Lleida**
Pieris napi	1		1		1	mobile		closed		LC		428	1.0			4.0
Polyommatus icarus	1		1			mobile		open	0.5	LC		150	0.4	Gind	1	3.9
Carcharodus alceae	1	1	1			mobile				LC		14	0.0			3.0
Lasiommata megera			1			mobile				LC		48	0.1	Gind	1	2.1
Pyronia cecilia			1			low mob	0.5	open	0.5	LC		10	0.0			2.0
Pararge aegeria			1			mobile		closed		LC		342	0.8			1.8

Table D.2.4. Prioritisation criteria applied to species in La Seu, to select the candidates most appropriate for monitoring possible impacts of *Bt* or GMHT or maize on butterflies we prioritised species according to the highest exposure in the field, susceptibility to *Bt* or herbicide, species that could be expected to be most responsive to changes, protected species and species with most information regarding ecology and biology.

	STEP B.1 Exposure		**STEP B.2 Susceptibility to Bt toxin/Herbicide**		**STEP B.3 Responsiveness**				**STEP B.4 Conservation value**		**STEP B.5 Information ecology and biology**				
	Adult present both years	Larval host plant near maize	Herbicide	Bt	Mobility		Habitat preference		IUCN 2010		Availability information (refs WOK)		Indicator		
Species	score	score	score	Score	Type	score	Type	score	category	Score	Nref	score	Type	score	**RANK La Seu**
Aglais io	1	1		1	mobile		closed		LC		194	0.5	Bt	1	4.5
Maniola jurtina		1			low mob	0.5	open	0.5	LC		135	0.3	Gind	2	4.3
Coenonympha pamphilus	1	1			low mob	0.5	open	0.5	LC		77	0.2	Gind	1	4.2
Thymelicus acteon		1			low mob	0.5	open	0.5	NT	1	21	0.0	Gind	1	4.0
Aglais urticae		1		1	mobile		open	0.5	LC		227	0.5	Bt	1	4.0
Pieris napi	1	1		1	mobile		closed		LC		428	1.0			4.0
Polyommatus icarus	1	1			mobile		open	0.5	LC		150	0.4	Gind	1	3.9
Lycaena phlaeas	1	1			mobile		open	0.5	LC		84	0.2	Gind	1	3.7
Pyronia tithonus	1	1			low mob	0.5	open	0.5	LC		65	0.2			3.2
Lasiommata megera	1	1			mobile				LC		48	0.1	Gind	1	3.1
Melanargia lachesis	1	1			low mob	0.5	open	0.5	LC		12	0.0			3.0
Pyronia cecilia	1	1			low mob	0.5	open	0.5	LC		10	0.0			3.0
Pararge aegeria	1	1			mobile		closed		LC		342	0.8			2.8
Iphiclides feisthamelii		1			mobile		open	0.5	LC	1	12	0.0			2.5
Hipparchia fagi		1			low mob	0.5			NT*	1	8	0.0			2.5
Carcharodus alceae	1	1			mobile				LC		14	0.0			2.0
Carcharodus flocciferus					metapop	1.0			NT	1	9	0.0			2.0
Polygonia c-album		1			mobile		open	0.5			61	0.1			1.6

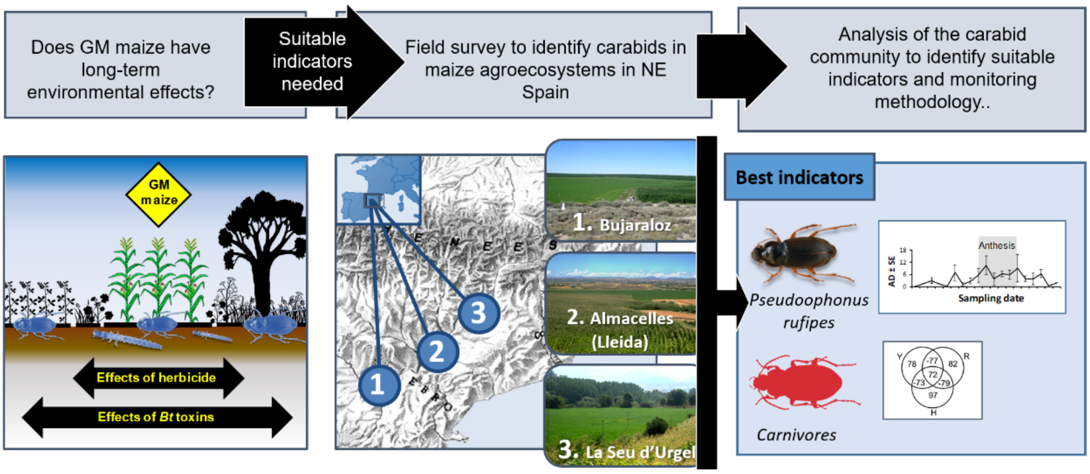
The following basic references were used to inform the selection process, in addition to field data obtained in the study. For further information, refer to the complete article.

1. **Exposure** of species to GM maize cultivation: Field data and butterfly phenology in the study area (García-Barros *et al.*, 2013)
2. **Susceptibility of butterfly species to *Bt* toxins** (Felke *et al.*, 2002, 2010; Lang & Vojtech, 2006; Kjær *et al.*, 2010; Lang & Otto, 2010; Schuppener *et al.*, 2012) **or to direct or indirect effects of modified herbicide** regimes (Pleasants & Oberhauser, 2012).
3. **Responsiveness** of species was considered according to mobility in the study area (Stefanescu *et al.*, 2011a), habitat specialisation (Herrando *et al.*, 2016), status as migrants (Stefanescu *et al.*, 2011b) or pest status (García-Barros *et al.*, 2013).
4. **Conservation value** of species according to the European IUCN red list (Van Swaay *et al.*, 2010) or the Spanish red list (Verdú *et al.*, 2011).

Information on ecology and biology. Availability of information was quantified by assessing the number of bibliographic references available on the Web of Science (WOS). Species particularly useful for monitoring GM maizes were European grassland indicator species(EEA, 2013) or species previously proposed for evironmental monitoring of *Bt* maize (Perry *et al.*, 2010, 2012; Holst *et al.*, 2013).

Chapter 3.

Monitoring carabid indicators could reveal environmental impacts of genetically modified maize



Monitoring carabid indicators could reveal environmental impacts of genetically modified maize

1. In Spain, where GM maize is cropped commercially, adverse effects could go undetected because postmarket monitoring is not based on relevant data from receiving agroecosystems. Monitoring carabids could improve detection capacity given exposure to GM cropping and sensitivity to environmental change. This study aimed to assess which, where and when carabid indicators should be sampled for improved postmarket monitoring.
2. Carabids were pitfall-trapped in maize agroecosystems (maize, field margins, alfalfa and semi-natural vegetation) across three regions of NE Spain, during two years.
3.Overall, 9,193 carabids of 42 species were identified, aggregated into trophic groups and used for calculating community measures. The best indicator was *Pseudoophonus rufipes* (De Geer), satisfying criteria of abundance, relevance, sensitivity and ease of sampling. The carnivore group should also be monitored as an indicator of biodiversity and invertebrate biological control.
4. The best sampling location was the field margin where carabid indicators are exposed to GM maize and abundant enough to require smaller sample sizes to detect population changes.
5. Finally, sampling should concentrate around maize pollen-shed because it is when carabid abundance is highest.
6. This study provides baseline data and shows that monitoring carabids could cost-effectively improve detection capacity of postmarket monitoring.

Key words: corn, risk assessment, pest management, genetic engineering, biotechnology, *Bt* maize, Herbicide Tolerant maize, ground beetle.

Genetically modified (GM) maize is cultivated on a commercial scale in Spain, where *Bt* maize (event MON810) occupied almost 132 thousand ha in 2014 (James, 2014). Although this is the only genetically modified (GM) crop produced in the EU, maize varieties containing other insect resistance (*Bt*) and herbicide tolerance (GMHT) traits are in the authorisation process. As a precautionary measure, EU legislation (Annex VII of Directive 2001/18/EC and Council Decision 2002/811/EC) requires post market environmental monitoring (PMEM) after placement on the market of any GM crop. As part of the PMEM, general surveillance (GS) aims to detect cumulative, delayed or unexpected adverse effects of GM crops on human health or the environment (EFSA 2006). This long-term monitoring is compulsory even if the environmental risk assessment identified no potential risks. Currently, GS guidelines (EFSA, 2011, 2014) recommend using three approaches: (1) monitoring the crop and its cultivation sites through a farm questionnaire; (2) using data collected by existing environmental surveillance networks and (3) analysing data from scientific literature. Implementation of a specific plan to monitor for adverse effects of GM crops is not contemplated in EU legislation despite the fact that farm questionnaires could not reveal changes in overall biodiversity; many agricultural areas lack appropriate environmental surveillance networks and scientific literature may not focus on relevant indicators. If GS is to serve its purpose, it should include indicators capable of revealing adverse effects of GM crops on ecosystem biodiversity and functions.

Carabids (Coleoptera: Carabidae) are widely used as indicators because they respond to environmental change (Rainio & Niemelä, 2003) and they are easy to collect by pitfall-trapping (Kotze *et al.*, 2011). They are particularly suitable for monitoring environmental effects of GM maize because they feed and breed within crop fields (Kromp, 1999), being more exposed to GM crops than other indicators proposed for this end, such as butterflies (Perry *et al.*, 2010). Moreover, because of their mobility, within-field impacts could carry over to neighbouring habitats. They are taxonomically diverse and provide important ecosystem services through control of invertebrate pests (Kromp, 1999) and weed seeds (Bohan *et al.*, 2011; Honek *et al.*, 2006). Their wide distribution and abundance in agricultural systems (Holland & Luff, 2000) allow for statistical analysis (Comas *et al.*, 2015; Legendre & Legendre, 1998). Finally, their taxonomy and ecology are sufficiently well known for identification to be relatively easy and to permit population changes to be interpreted (Kotze *et al.*, 2011; Rainio & Niemelä, 2003).

Carabids respond to agricultural management (Aviron *et al.*, 2005; Döring & Kromp, 2003; Holland & Luff, 2000; Legrand *et al.*, 2011) so they can be expected to respond to GM cropping through several mechanisms detailed below. Although GS is not hypothesis driven (EFSA, 2011), likely exposure pathways to GMHT and *Bt* maize should be evaluated to select appropriate indicators.

The main mechanism through which cultivation of GMHT maize may affect carabids is through changes in herbicide type, timing and number of applications compared to cultivation of conventional maize (Brooks *et al.*, 2005; Heard *et al.*, 2006). These changes could affect carabids directly through herbicide toxicity, which is very rarely reported in the literature (Brooks *et al.*, 2005; Michalková & Pekár, 2009). Alternatively, the impact may be indirect, due to shifts in weed density, phenology, distribution and composition (Heard *et al.* 2006; Smith *et al.*, 2008; Albajes *et al.* 2009), as weeds provide food resources and control microclimate for carabids (Holland & Luff, 2000). Such changes in weed populations and assemblages affect carabids differently according to their feeding preferences (Purtauf *et al.*, 2005; Vanbergen *et al.*, 2010). For instance, in the UK Farm Scale Evaluation of GM crops (Perry *et al.* 2003) higher weed density in GMHT maize increased numbers of seed feeding carabids (Brooks *et al.*, 2003; Heard *et al.*, 2006). This effect on seed feeding carabids was maintained when different herbicide regimes were assessed (Brooks *et al.*, 2005). In a four-year study, Albajes *et al.* (2011) indicated that moderate changes in herbicide regimes did not affect predator densities despite lower weed densities in GMHT maize and only dramatic weed alteration affected abundance and composition of predatory fauna. Indeed, in conventional maize broad spectrum herbicides can only be applied prior to crop emergence, when the early removal of all plant cover may hinder colonisation by carabid predators (Brooks *et al.*, 2005). This can result in lower biological control of insect pests regarding GMHT maize, where broad spectrum herbicides may be applied later in the season (Bigler & Albajes, 2011).

Bacillus thuringiensis (*Bt)* crops produce taxon-specific insecticidal Cry proteins, toxic by ingestion (Gill *et al.*, 1992). Thus, organisms taxonomically close to the target insects (in maize Lepidoptera and Coleoptera) are potentially more susceptible. Exposure to *Bt* toxins is greatest for species feeding on GM maize but carabids may also be exposed by accidental ingestion of pollen (Lepping, 2009; Stanley-Horn *et al.*, 2001); exposure to root exudates (Icoz & Stotzky, 2008), consumption of contaminated prey (Obrist *et al.*, 2006) or lower numbers and quality of prey (Meissle *et al.*, 2005; Naranjo, 2009). So far however, Lepidoptera-resistant maize had no detectable effects on carabids in the field (Comas *et al.*, 2014; De La Poza *et al.*, 2005; Kocourek *et al.*, 2013; Lopez *et al.*, 2005) although Meissle *et al.* (2005) detected adverse effects on carabids fed with larvae that had ingested *Bt* toxins. The situation was similar for Coleoptera-resistant maize, most studies found no effects (Lepping, 2009; Leslie *et al.*, 2010) although one study found a reduction in overall carabid numbers but the mechanism was unknown (Stephens *et al.*, 2012). However, the lack of consistent adverse environmental effects of *Bt* maize could simply indicate that the timescale of these studies was insufficient to detect subtle population effects that may only be revealed after decades of generalised cultivation.

In previous field trials carried out in NE Spain (Albajes *et al.*, 2009; Eizaguirre *et al.*, 2006), the carabid group was abundant enough to detect population changes in *Bt* maize at low sample sizes (Albajes *et al.*, 2013; Comas *et al.*, 2013). Nevertheless, using overall carabid number for monitoring does not reveal how GM cropping may be affecting carabid diversity or the ecosystem functions they carry out.

This study outlines an improved environmental monitoring plan that would make GS more likely to detect potential adverse effects of *Bt* and GMHT maize cultivation on ecosystem diversity and functions. The specific aims were: i) to assess abundance and frequency of carabid species present in the maize agroecosystem across regions and years, providing baseline information; ii) to determine the most suitable carabid indicators for standardised monitoring based on distribution, statistical power, sample size, and capacity for reflecting ecosystem diversity and functions; and finally; iii) to determine the most suitable sampling sites and dates among those tested in this work.

Materials and methods

Study regions

Carabids were sampled in three different maize cropping regions in NE Spain to account for variability due to differences in landscape, cultural practices, and agroclimatic conditions. Bujaraloz study region (41°29′50″N 0°9′13″O) is located in the Monegros badlands at an altitude of 350 m; Almacelles (41°43′57″N 0°26′25″E) is at 250 m in the Lleida agricultural plains; and finally, La Seu, (42°21′32″N 1°27′43″E), is at 691 m in the Pyrenees. Climate and cultivation practices are similar in Bujaraloz and Almacelles where maize is cultivated for grain, here, intensive soil cultivation, applications of pre-emergence herbicides and deployment of *Bt* maize (event MON810) for cornborer control are common practice. Almacelles landscape is a mosaic of arable crops and orchards; semi-natural vegetation is confined to hills, waterways and paths. In Bujaraloz fields are larger and landscape is composed of arable crops and large uncultivated patches. Finally, in La Seu, maize is cropped for silage as part of a yearly crop rotation; agricultural practices include no-till and pre-emergence herbicide applications. *Bt* maize is not used as there is no cornborer pressure; landscape is a mosaic of forage crops, pastures and forest. Average maize field sizes were 8.4 ± 1.45 ha in Bujaraloz, 5.4 ± 0.9 ha in Almacelles and 3 ± 0.7 ha in La Seu.

Carabid sampling and identification

Ten conventionally managed maize fields (5 in 2011 and 5 more in 2012) were sampled in each study region (Bujaraloz, Almacelles and La Seu). Three neighbouring habitats were sampled when present (29 field margins, 27 alfalfa fields and 19 areas of semi-natural vegetation). Each field and its neighbouring habitats were sampled three times: in July during maize vegetative stages (V10-V14), in August around pollen-shed (VT-R1) and in September at ripening of the grain (R4-R6). A line of three pitfall traps, 10 m between them, was placed in the sampling site 15-20 m from the edge, where possible. Only three traps were used because this study aimed to outline a feasible monitoring plan and this number of traps had previously been used successfully by our research group (De La Poza *et al.*, 2005). Traps consisted of a buried plastic sheath in which a glass jar (Ø 9 cm, 17 cm deep) was placed flush with the soil surface and filled with 250 mL of a 20 % solution of propylene glycol and water, covered with a polystyrene tray placed 2 cm above the ground to prevent flooding and reduce vertebrate bycatch. Traps were left active during one week each time and covered when not in use.

Carabids were identified following literature (mainly Luff 2007 and Trautner & Geigenmüller 1987), nomenclature followed Serrano (2003). After expert revision by E. Vives, voucher specimens were stored at Lleida University. Trophic groups, indicating ecosystem function, were assigned according to literature (Ameixa & Kindlmann, 2008; Larochelle, 1990; Purtauf *et al.*, 2005; Vanbergen *et al.*, 2010). Criteria for assigning trophic groups* were reports of adult beetles feeding solely on animal material (carnivores), solely on plant material (phytophages) or on both (omnivores).

In pitfall trapping, the abundances of a species in a trap are influenced not only by the abundance of the species in the environment but also by its activity (Honek, 1988). Therefore the abundance of the carabid species collected by this method is only a proxy of the true composition and size of carabid populations. Nevertheless, for simplicity, hereafter the term “abundance” will be used throughout this study to refer to the number of carabids collected in pitfall traps.

*Note: all species assigned to the same trophic group were summed for each sampling date and site and then calculations were performed the same as for single species.

Carabid community measures

Species richness and Shannon’s diversity index were calculated for each plot, in order to determine the potential value of carabids as biodiversity indicators. As the observed species richness is a function of sample size (Gotelli & Colwell, 2001), Chao 1 index was used to estimate true species richness (Chao, 1987), which is the predicted value considering number of unrecorded but present species (Colwell, 2013). Similarity of carabid assemblages between habitats, was calculated using the estimated abundance-based Chao-Jaccard similarity index (Chao *et al.*, 2005). This was done in order to assess the similarity of maize carabid assemblages to assemblages in other habitats. In this way, information can be obtained on the suitability of monitoring the impacts of GM maize in habitats other than maize. The software used for these calculations was EstimateS 9.1 (Colwell, 2013).

Linear relationships between carabid species' and groups' abundance to community measures were tested by Pearson's correlation coefficient, to evaluate their potential as indicators of carabid diversity (Duelli & Obrist, 2003).

Frequency, proportion of fields where a species was present, and dominance, species together constituting 95 % of relative abundance (Luff, 2002), were calculated to identify the most common and abundant species. These species are potentially the most valuable for use as standardised indicators of GM maize impacts.

Habitat, region and year as sources of baseline variability

For each habitat and sampling site, mean values of community measures and mean abundance of carabid species and trophic groups were calculated. The effects of the main factors habitat, region and year and their interactions on these indicators were assessed to obtain information on the sources of variability of baseline carabid data. Data were analysed with a generalised linear model (GLM), using a Poisson distribution and log-link function (Gaussian distribution and identity-link function for community measures). As the interactions between the main factors were significant for most carabids, further analyses were carried out in two steps.

In the first step, variation partitioning was performed to determine how much of the variation of the final GLM model was explained by the pure effect of each factor (habitat, region and year) and which proportion was attributable to their shared effect (Legendre & Legendre, 1998; Whittaker, 1984). Variation partitioning was carried out with the VarPart function in the R package ModEva (Barbosa *et al.* 2014), and calculated by using the squared value of Spearman's correlation coefficient between the values of the final model and a model based only on the pure factor. Negative values represent opposing effects.

The goodness of fit statistic, analogous to r^2, representing the proportion of deviance explained by the GLM model was approximated by $D^2 = 1 - [residual\ deviance \div null\ deviance]$ (Yee & Mitchell, 1991).

In the second step, regional and year-to-year variation was examined within each habitat (30 maize fields, 29 field margins and 27 alfalfa plots); the semi-natural habitat was not analysed due to low carabid abundance. Significant differences were determined by a chi-square test ($\alpha = 0.05$) and explored using Tukey HSD. Analyses were carried out with R (R Core Team, 2013) using the MASS package (Venables & Ripley, 2002).

Long-term population fluctuation of common carabids

Historic data from 2005 to 2012 obtained by our research group from field studies on non-target effects of GM maize carried out in the Lleida plains (see e.g. Comas *et al.*, 2014), where Almacelles is located, were used to determine the dominant species of the carabid community and their population fluctuations in a longer time-series. Sampling followed a similar methodology (2-3 pitfall traps in each maize plot and 3-8 sampling dates) but only the most abundant species were identified. Mean carabid abundance and SD were calculated for each sampling date from May to October. Results were used to determine if dominant species' composition and population peaks could also be identified in a 2-year study such as this one.

Power analysis to determine required sample sizes

Prospective power analyses were carried out to determine sample sizes (number of paired sites of GM vs. non-GM crop) needed to detect a change in carabid populations using a two tailed t-test (population decrease or increase). The probability of committing a type I error (α) was set at 0.05 and type II error (β) was set at 0.2, ($statistical\ power = [1 - \beta] = 0.8$). The statistical power measures the chance of detecting an effect of a known magnitude using a specified experimental design, and varies according to the magnitude of the effect, set at a 30 % change regarding the comparator population, considered adequate for studies on effects on non-target organisms (Lang & Bühler, 2012; Perry *et al.*, 2003). Comparator populations were approximated by calculating average carabid abundance in each habitat type, within each region. Data were transformed by $\log_{10}$ (x+1) when necessary for normalization; the software used was JMP11 (JMP®, 2013).

Results

Carabids in maize fields

In the 30 maize fields sampled across the three study regions, 2,368 individuals belonging to 34 species were collected. Table 1 shows mean abundance of each species per trap and week, allowing comparison with captures in other studies. In general the assemblages were species-poor and uneven, dominated (95 % total catch) by a few very abundant species, characteristic of arable systems with a high level of disturbance. Species common to maize fields across the different study regions have the greatest potential for use as standardised indicators for monitoring impacts of GM maize. There were only seven species common to maize across the three regions, from most abundant to least these were *Pseudoophonus rufipes* (DeGeer), *Poecilus cupreus* (L.), *Bembidion lampros* (Herbst), *Pterostichus niger* (Schaller), *Brachinus (Brachynidius) sclopeta* (Fabricius), *Clivina fossor* (L.) and *Brachinus crepitans* (L.). When aggregated into trophic groups, 70 % of maize carabids were omnivores, 26 % were obligate carnivores and only 4 % were obligate phytophages.

Estimated species richness (Chao 1 mean ± SD) was low, ranging from 18.5 ± 2.6 in Almacelles, 17.5 ± 1.3 in Bujaraloz, to 25.0 ± 5.5 in La Seu. Shannon diversity index was 1.13 in Bujaraloz, 1.61 in Almacelles and 1.96 in La Seu. There were a few weak correlations between community measures and abundance of single species or trophic groups, indicating that abundance of most species and trophic groups would be poor indicators of biodiversity. Only *B. lampros* and carnivores correlated to species richness (Pearson's correlation coefficient 0.43 and 0.5 respectively); Shannon index and *B. sclopeta* correlated positively and *P. rufipes* negatively (Pearson's correlation coefficient 0.4 and -0.42 respectively).

Table 1. Carabid species collected from pitfall traps in maize fields (n) in three regions of NE Spain. The table shows species' mean seasonal captures (abundance, AD) ± SE and frequency (F) per trap. **In bold, species common to all regions.**

Species in maize	TG	Bujaraloz (n = 10)		Almacelles (n = 10)		La Seu (n = 10)	
		AD ± SE	F	AD ± SE	F	AD ± SE	F
Agonum muelleri	C	0	-	0	-	0.0±0.02	0.3
Amara crenata	P	0	-	0	-	0.0±0.01	0.1
Amara montivaga	P	0	-	0	-	0.0±0.03	0.1
Ancholeus nitidus	C	0.0 ± 0.04	0.1	0	-	0	-
Ancholeus puncticollis	C	0	-	0.0 ± 0.01	0.1	0	-
Anchomenus dorsalis	C	0.6 ± 0.25	0.8	2.4 ± 1.06	1	0	-
Badister unipustulatus	C	0.0 ± 0.01	0.1	0	-	0	-
Bembidion ambiguum	C	0.1 ± 0.08	0.2	0	-	0	-
Bembidion guttula	C	0	-	0.0 ± 0.02	0.1	0	-
Bembidion lampros	**C**	**0.5 ± 0.30**	**0.7**	**0.3 ± 0.20**	**0.3**	**0.7 ± 0.37**	**0.5**
Bembidion quadrimaculatum	C	0.2 ± 0.10	0.4	0	-	0	-
Brachinus crepitans	**C**	**0.0 ± 0.01**	**0.1**	**0.1 ± 0.04**	**0.4**	**0.0 ± 0.01**	**0.1**
Brachinus sclopeta	**C**	**0.1 ± 0.04**	**0.2**	**0.0 ± 0.02**	**0.2**	**0.2 ± 0.18**	**0.1**
Calathus ambiguus	C	0	-	0.6 ± 0.43	0.5	0.0 ± 0.01	0.1
Calathus fuscipes	O	0	-	0	-	2.1 ± 0.89	0.9
Calathus melanocephalus	C	0	-	0	-	0.0 ± 0.01	0.1
Calathus rotundicollis	C	0	-	0	-	0.0 ± 0.01	0.2
Calosoma maderae	C	0.1 ± 0.05	0.3	0	-	0	-
Carabus violaceus	C	0	-	0	-	0.0 ± 0.02	0.1
Clivina fossor	**C**	**0.0 ± 0.02**	**0.2**	**0.1 ± 0.03**	**0.3**	**0.1 ± 0.04**	**0.3**
Cylindera paludosa	C	0	-	0.0 ± 0.01	0.1	0	-
Harpalus atratus	P	0.1 ± 0.06	0.1	0.0 ± 0.01	0.1	0	-
Harpalus distinguendus	O	0	-	0.3 ± 0.28	0.5	0.0 ± 0.03	0.1
Harpalus serripes	P	0	-	0	-	0.5 ± 0.48	0.1
Harpalus sulphuripes	P	0	-	0	-	0.0 ± 0.01	0.1
Poecilus cupreus	**O**	**0.3 ± 0.32**	**0.3**	**3.0 ± 1.36**	**0.8**	**1.1 ± 0.50**	**0.9**
Poecilus purpurascens	O	0	-	0	-	0.5 ± 0.45	0.1
Pseudoophonus calceatus	O	0	-	0.0 ± 0.01	0.1	0	-
Pseudoophonus rufipes	**O**	**7.7 ± 2.80**	**1**	**4.4 ± 2.01**	**0.7**	**1.2 ± 0.36**	**1**
Pterostichus niger	**C**	**0.5 ± 0.44**	**0.4**	**0.0 ± 0.03**	**0.1**	**0.1 ± 0.04**	**0.4**
Pterostichus vernalis	C	0	-	0	-	0.0 ± 0.01	0.1
Syntomus obscuroguttatus	C	0.0 ± 0.02	0.3	0	-	0	-
Tachys bistriatus	C	0.0 ± 0.02	0.1	0.1 ± 0.05	0.2	0	-
Trechus quadristriatus	C	0.1 ± 0.04	0.3	0.0 ± 0.02	0.3	0	-

Trophic groups (TG) are carnivore (C), omnivore (O) or phytophage (P).

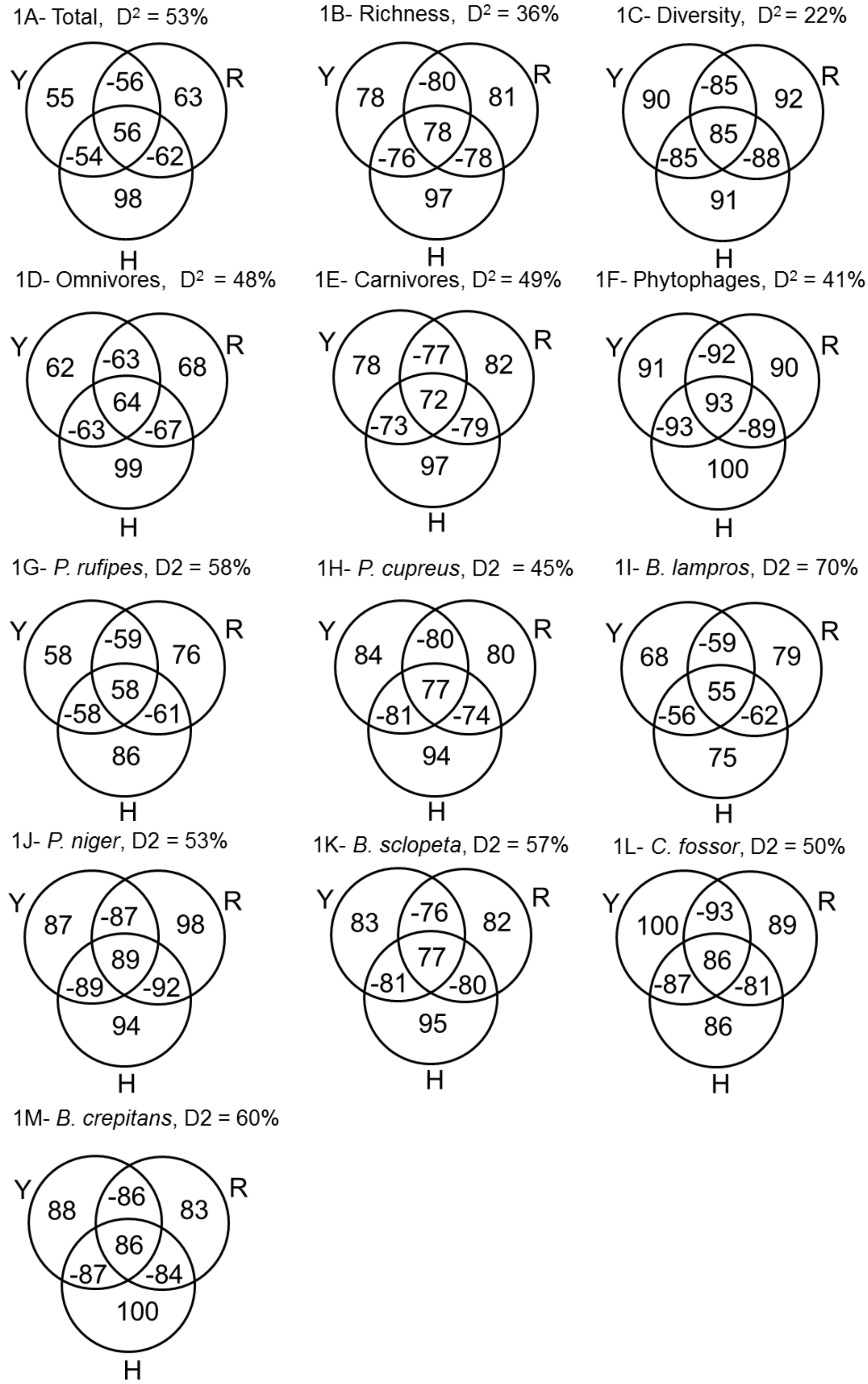
Carabids in neighbouring habitats

Within each region, similarity of carabid assemblages captured in maize to those captured in neighbouring habitats (field margins, alfalfa and semi-natural vegetation) was assessed in order to determine if maize carabids were present in other habitats and where these could best be sampled. The highest number of shared species was observed between maize and field margins; 15, 12 and 14 species in Bujaraloz, Almacelles and La Seu respectively. Nevertheless, similarity indexes revealed most similar habitats were maize fields and their margins in Bujaraloz (0.94) and La Seu (0.96), whereas in Almacelles (0.88) alfalfa was most similar to maize (0.90). The habitat least similar to maize was consistently the semi-natural vegetation, probably due to the lower perturbation of natural systems and lower soil humidity compared to irrigated agricultural land; similarity indices were 0.7, 0.57 and 0.27, in Bujaraloz, Almacelles and La Seu, respectively. In maize field margins 4,938 individuals of 42 species were collected (Annex 1). In alfalfa 1,689 individuals of 30 species were collected (Annex 2). In semi-natural vegetation neighbouring maize plots, only 198 specimens were captured belonging to 30 species (Annex 3).

Contribution of habitat, region and year to overall variability

Habitat, region and year contributed to the baseline variability of the carabid data from maize agroecosystems in NE Spain. Variance partitioning revealed that habitat identity explained the largest proportion of explained variability in the carabid community with distinct assemblages in each maize or alfalfa fields, at field margins and in semi-natural vegetation (Fig. 1). After habitat type, there were substantial regional (Bujaraloz vs Almacelles vs La Seu) and annual (2011 and 2012) differences, suggesting spatial and temporal turnover in the species pool. The main predictor (habitat, region or year) accounted for 100 % of the variation in some cases indicating that the addition of further factors could not improve the explanatory capacity of the model. The estimated proportion of variability explained by the GLM model, D^2, was above 40 % for all dependent variables but for the community measures for which it was lower (Fig 1B-C).

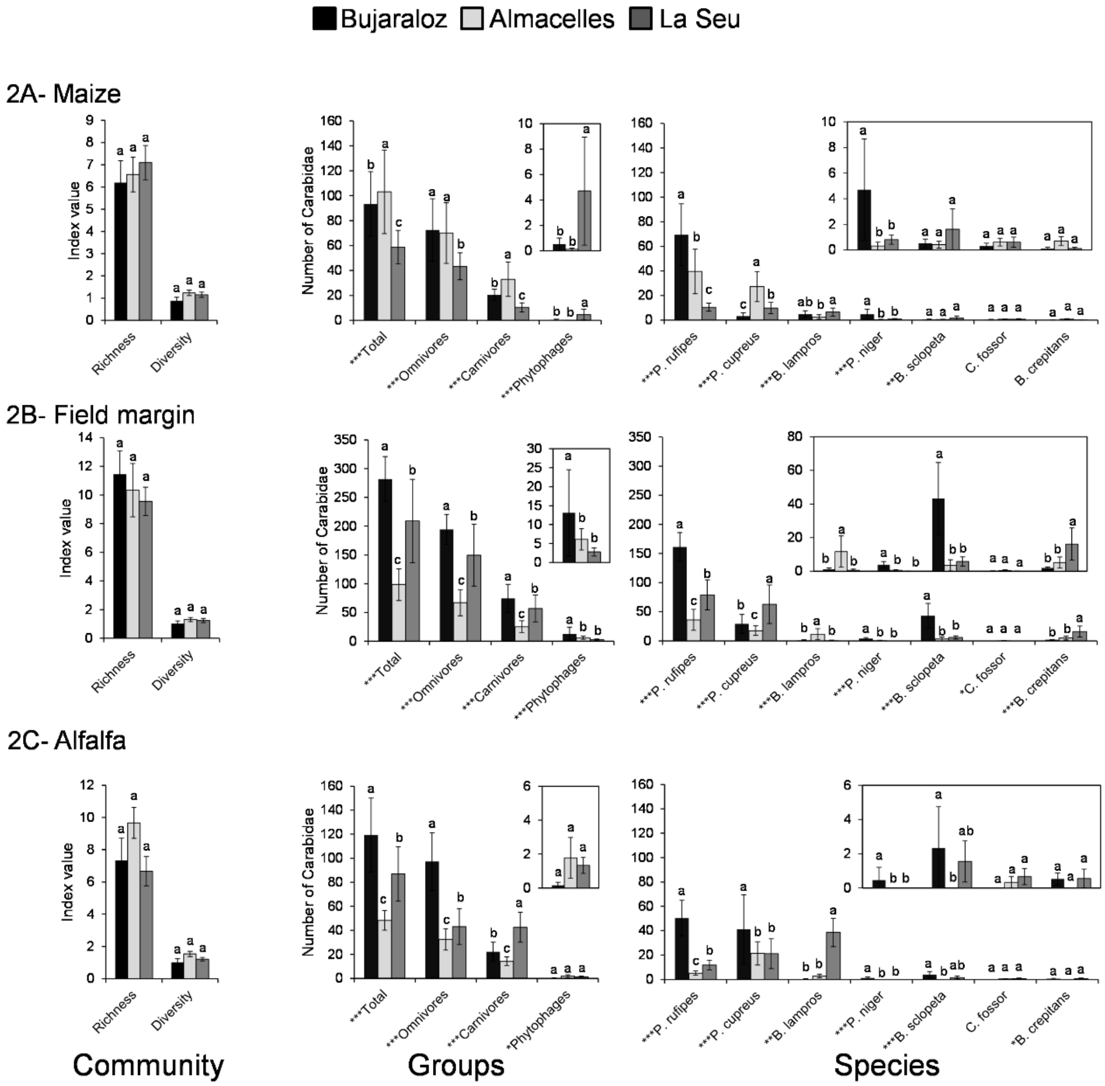
Figure 1. Variation partitioning of carabids among explanatory variables: region (R), habitat (H) and year (Y). The variability accounted for by the model is approximated by D^2, and the proportion of variability explained by each factor and interaction between factors is shown for overall carabid number (1A), Chao 1 estimated species richness (1B), Shannon diversity index (1C), trophic groups (1D - F) and the seven ubiquitous species (1G - M). Explanatory note: the numbers in the circles represent variability explained by each factor, intersections represent the variability explained by a model containing various variables, negative values mean that the model with both variable explains less variability the single variable models.



When regional and year-to-year variation of the dependent variables was examined within each habitat, there were differences between regions (Fig. 2) and years in abundance of most groups (overall carabids, omnivores, and carnivores) and the seven ubiquitous maize species (*P. rufipes, P. cupreus, B. lampros, P. niger, B. sclopeta, C. fossor* and *B. crepitans*), but community measures were not sensitive enough to reflect these differences. Differences between regions followed no general tendency in any of the three habitats considered, which is to be expected from three regions with differing agrienvironmental contexts. In maize (Fig 2A), overall carabid abundance was highest in Almacelles, possibly due to higher weediness of maize. In field margins (Fig. 2B) and alfalfa (Fig 2C), carabids were most abundant in Bujaraloz where landscape is more open than that of La Seu, and less altered than that of Almacelles. Omnivores were always the most abundant trophic group, followed by carnivores and phytophages.

Regarding the year-to-year variation in maize, carabids were clearly more abundant in 2011 (100 ± 24.72) than in 2012 (70 ± 15.99) (χ^2_{28}= 1863.3, $P < 0.001$), a tendency followed by most groups and common species. Conversely, in field margins carabids were more abundant in 2012 (233 ± 49.29) than in 2011 (164 ± 39.29) (χ^2_{27}=4144.7, $P < 0.001$), and this was also true for omnivores and carnivores but not for phytophages. In alfalfa there were no differences in carabid abundance between 2011 (78 ± 12.44) and 2012 (85 ± 20.09) (χ^2_{22}= 1054.2, $P = 0.058$) because omnivores were more abundant in 2012 and carnivores and phytophages in 2011.

Figure 2. Regional (explanatory variable) differences between carabid community measures, abundance of groups and ubiquitous maize species (response variables in the x axis) in three different habitats: maize, maize field margins and alfalfa. Analysis was carried out using a GLM, Poisson distribution and log-link function for count data and Gaussian distribution and identity-link function for community measures. Differences were determined by a Chi-square test. Significant differences were explored by HSD and are indicated with different letters, significance (α = 0.05) is indicated following indicator by *$p < 0.05$, **$p < 0.01$ and $p < 0.001$. Df for community measures and abundance data, respectively: 23 and 24 for maize, 20 and 23 for margins; 17 and 18 for alfalfa.



Common carabids in maize fields

Considering historical data from 2005 to 2012, *P .rufipes* clearly dominated the assemblage in maize fields in Lleida; proportions were: *P. rufipes* 48 %, *P. cupreus* 24 %, *Anchomenus dorsalis* (Pontoppidan) 7 %, *Bembidion* spp. 4 %, *Brachinus* spp. 1 % and *Harpalus distinguendus* (Duftschmid) 1%. Mean abundance was very variable across fields and years (see SE bars in Fig. 3); however, the overall picture is similar to the results from Almacelles in 2011 and 2012, indicating that identification of dominant species may not need prolonged sampling. The most abundant species, *P. rufipes*, *P. cupreus* and *A. dorsalis*, peaked around pollen-shed, indicating that this could be a good time for sampling.

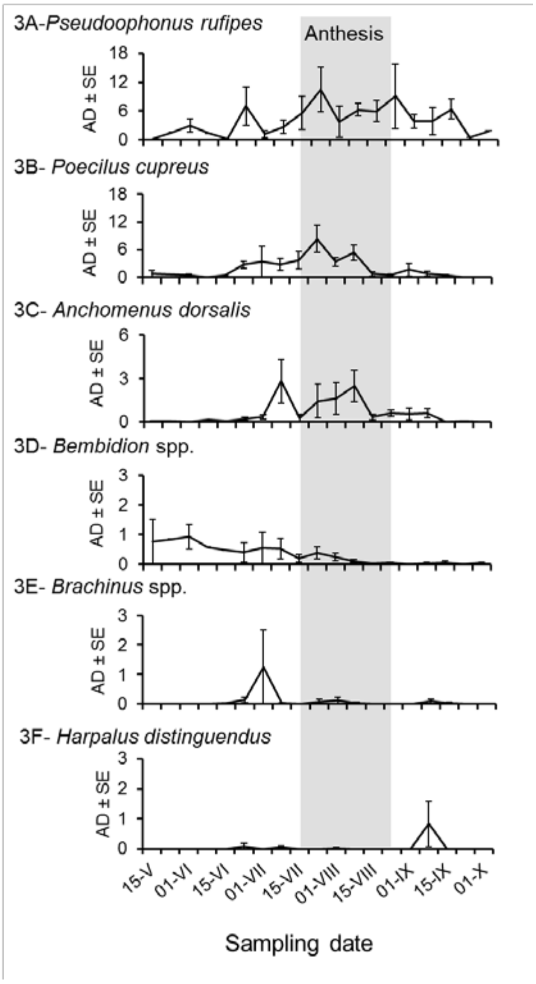


Figure 3. Population fluctuation of the most common carabid taxa in Lleida plains during the maize growth cycle, maize pollen-shed period is highlighted in grey. The graphs show weekly mean captures per trap (activity-density ± SE), calculated by pooling historical data from 2005 to 2012

Power: sample sizes required to detect GM effects

Sample sizes needed to detect a 30 % carabid population change (pairs of maize fields or margins) are shown in Table 2. Lowest sample sizes were obtained using overall carabid abundance (2-10 site pairs), closely followed by the observed (3-6 site pairs) or estimated (2-6 site pairs) species richness. When aggregated into trophic groups, sample sizes required were also low for omnivores (3-16 site pairs) and carnivores (9-46 site pairs) but very high for phytophages (77-1,571 site pairs), due to their relative rarity. Regarding single species, *P. rufipes* would require least site pairs (3-46), followed by *P. cupreus* (26-394 site pairs) and *B. sclopeta* (33-884 site pairs). Other species required fewer samples but were not present in maize across regions. Sampling sizes to detect population change were generally lower in field margins than in maize fields. In alfalfa (Annex 4), the lowest sample size to detect population change, using *P. cupreus*, was too high for a feasible monitoring plan.

Table 2. Sample sizes (number of pairs of maize fields or margins) needed to detect a 30 % change in carabid populations. Calculations are based on means and SD for both 2011 and 2012 combined. Data were transformed by ($\log_{10}$ (x+1)) where necessary for normalization. A pairwise t-test for independent data was used, assuming equal number of units in each group and two-tailed test, statistical power was set at 80 % (1-β = 0.8) and significance level α at 0.05.

Assessment endpoint	**Bujaraloz**		**Almacelles**		**La Seu**	
	Maize	**Margin**	**Maize**	**Margin**	**Maize**	**Margin**
All carabids	6	2	10	10	8	8
Diversity indices						
Richness (Sobs)	4	5	6	3	3	4
Richness (Chao 1)	5	5	6	2	3	4
Diversity (Shannon)	7	30	18	8	7	7
Trophic groups						
Omnivores	11	3	16	15	11	10
Carnivores	9	13	22	21	46	37
Phytophages	567	77	1571	143	253	103
Common maize species						
Pseudoophonus rufipes	12	3	28	21	46	24
Poecilus cupreus	394	42	32	26	81	52
Anchomenus dorsalis	105	49	38	30		631
Calathus fuscipes					48	48
Bembidion lampros	117	267	394	951	120	84
Calathus ambiguus		1732	131	3078	1571	924
Pterostichus niger		164	1571		394	717
Harpalus serripes					253	271
Poecilus purpurascens					334	
Harpalus distinguendus		269	319	107	1571	309
Brachinus sclopeta	394	33	884	121	771	318
Bembidion quadrimaculatum	394	1194				
Clivina fossor	567	3078	394		615	1006

Common maize species are ordered according to mean abundance.

Discussion

Unsurprisingly, species identity and abundance differed between regions, although they were located in the same geographic area. This was an expected outcome as the study regions had been selected because of the agrienvironmental differences. For example, differences in the surrounding landscape in the three regions could have affected the composition of carabid populations (Vanbergen *et al.*, 2010). Similarly, differences in agricultural management in the three areas could have also influenced composition and abundance of carabid populations (i.e. Holland & Luff, 2000). This highlights the need to test indicator species across a wide geographic area (Büchs, 2003) to ensure that small differences in landscape, land management or climatic context do not reduce the efficacy of the indicator to monitor potential GM maize impacts. Conversely, despite differences in carabid abundance between years, there were few changes in composition of the most abundant species (Ortego *et al.*, 2009 and this study). In view of this, the most abundant or frequent species in a region may be identified in few seasons if sampling a large number of sites.

The most cost-effective option is monitoring few, easily identified taxa. The only species common to all regions, also frequent and abundant across sites, was *P. rufipes*. This species attains sufficient abundance that it is suited to be a focus of future monitoring because the number of sites required is realistic. As it is also abundant in maize elsewhere in Europe (Kocourek *et al.*, 2013; Smith *et al.*; 2008) it could prove useful as an indicator across the EU (Büchs, 2003). Although *P. rufipes* is omnivorous, it is a good indicator of carabid biodiversity (Döring & Kromp, 2003) and is economically important, preying on invertebrates (Jörgensen & Toft, 1997) and weed seeds (Harrison & Gallandt, 2012; Shearin *et al.*, 2008). Populations of *P. rufipes* are correlated to larger spring-germinating weed seeds (Brooks *et al.*, 2012), the main weeds in maize. Reduced weed abundance due to GMHT cropping could result in a drop in *P. rufipes* populations (Döring & Kromp, 2003; Eyre *et al.*, 2013). As it reproduces and hibernates in the crop field (Luff, 1980) both adults and larvae are exposed to *Bt* toxins in soil. Finally, identification does not require great expertise as it is a large species and there were no congeners leading to misidentification.

Using trophic group indicators allows quantifying the impact of changes in landscape or agricultural practices on ecological function (Purtauf *et al.*, 2005; Vanbergen *et al.*, 2010) despite differences in species’ identity. Phytophages may ingest toxins by feeding on *Bt* maize materials and they depend directly on resources provided by the weeds affected by cultivation of GMHT varieties. Moreover, they are very sensitive to environmental change (Purtauf *et al.*, 2005; Vanbergen *et al.*, 2010; Woodcock *et al.*, 2010). Nevertheless, phytophage carabids' abundance was low, requiring sample sizes that were too high to be practicable for monitoring.

The main exposure pathway of carnivores to GM maize would be through prey (Meissle, 2005). As they are at the top of the trophic web, they integrate a substantial amount of ecological information from the maize community. They would be the best indicator of biodiversity and of invertebrate biological control function, and the sample sizes to detect differences would be low enough for practical monitoring.

Omnivores are exposed through the mechanisms described for both phytophages and carnivores. Although they are considered to be less sensitive to environmental change than carnivores or phytophages (Purtauf *et al.*, 2005), they also respond to habitat alteration (Eyre *et al.*, 2013) and contribute to biological control. They were the most abundant trophic group and therefore sample sizes for detecting changes would be relatively smaller.

Carabid diversity measures are widely used as environmental indicators (Duelli & Obrist, 2003; Heink & Kowarik, 2010) and for detecting differences between farming systems (Holland & Luff, 2000). Indeed, in this study the lowest sample sizes were found using diversity measures but these were unable to reflect differences in species' identity and abundance existing between regions and years; possibly due to the low trap number and sampling window used per site. In these conditions, diversity measures are not sensitive enough for monitoring and they are not recommendable in PMEM.

Carabid communities are characteristic of each habitat (Smith *et al.*, 2008). Nevertheless, as maize shared many species with other habitats as recorded in this study and some others conducted in the area (Núñez, 1999; Madeira & Pons, 2015;), impacts in GM maize fields would influence other communities. Indeed, species ubiquitous to maize were also present in field margins where sample sizes needed to detect changes were generally lower. Margins play an important role for carabid conservation in agricultural systems (Holland & Luff, 2000), as well as being closely exposed to GM cropping (Bethwell *et al.*, 2012; Roy *et al.*, 2003). Thus, exposure to *Bt* maize pollen deposition or increased herbicide drift due to GMHT maize cultivation could reduce carabid diversity or biological control functions in surrounding habitats. Finally, placement and access to traps is considerably easier than in maize fields.

Environmental monitoring is time consuming and costly so the number of traps and sampling dates should be reduced when possible. This study shows that using only three pitfall traps per sampling location could reveal population changes with low sample sizes for some species. Sampling date was not addressed specifically in this study as it has been recently discussed in depth (Comas *et al.* 2015). Therein, as in this study, authors found carabid catches were most abundant around maize pollen-shed. Relative variability decreased as abundance of taxa increased, so they recommended using sampling dates with the greatest abundance as long as the number of individual samples was sufficient to warrant a low sample size to detect changes in population numbers.

Conclusions and recommendations for monitoring

This study contributes to design a PMEM plan for detecting impacts of GMHT or *Bt* maize and additionally it provides baseline data on carabids that are valuable for monitoring effects of natural or anthropogenic changes on maize agroecosystems. Overall, *Pseudoophonus rufipes* is the best indicator species, satisfying criteria of abundance, relevance, sensitivity and ease of sampling. However, the carnivore group should also be included as indicator of invertebrate biological control and of biodiversity. The field margin is the best sampling location as this habitat is in close contact with the GM maize crops, it is exposed to GMHT and GM*Bt* effects and it shares many species with maize that are abundant enough to require lower sample sizes to detect population changes. Finally, sampling should concentrate around pollen shed because it is when carabid abundance is highest. The results obtained in this study show that carabids may be useful for post market environmental monitoring purposes and provides some recommendations to improve practicability of PMEM without losing detection capacity.

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Supplementary material Chapter 3

Annex 1. Carabids in maize field margins.

Table A.1. Carabid species collected by pitfall trapping in margins of maize fields (n) in three regions of NE Spain. The table shows species' mean seasonal captures (AD) ± SE and frequency (F) per trap. In bold, species common to all regions.

Species in margins (part 1)	**TG**	**Bujaraloz (n = 10)**		**Almacelles (n = 10)**		**La Seu (n = 9)**	
		AD ± SE	**F**	**AD ± SE**	**F**	**AD ± SE**	**F**
Acinopus picipes	P	0.0±0.01	0.1	0	-	0	-
Agonum muelleri	C	0	-	0	-	0.2±0.13	0.4
Amara aenea	O	0	-	0	-	0.1±0.08	0.3
Amara brunnea	P	0	-	0	-	0.0±0.02	0.1
Amara communis	P	0	-	0	-	0.0±0.01	0.1
Amara montivaga	**P**	**1.1±1.01**	**0.4**	**0.1±0.08**	**0.2**	**0.0±0.01**	**0.1**
Ancholeus puncticollis	C	0.0±0.02	0.1	0	-	0	-
Anchomenus dorsalis	**C**	**1.7±0.61**	**0.9**	**3.6±1.75**	**0.8**	**0.0±0.03**	**0.2**
Badister unipustulatus	C	0.0±0.01	0.1	0.0±0.01	0.2	0	-
Bembidion ambiguum	C	0.0±0.04	0.1	0.0±0.02	0.1	0	-
Bembidion lampros	**C**	**0.1±0.07**	**0.5**	**0.1±0.07**	**0.1**	**1.3±1.04**	**0.7**
Bembidion quadrimaculatum	C	0.1±0.06	0.1	0	-	0	-
Brachinus crepitans	**C**	**0.2±0.06**	**0.7**	**1.8±1.06**	**0.5**	**0.6±0.37**	**0.4**
Brachinus sclopeta	**C**	**4.8±2.38**	**0.5**	**0.6±0.30**	**0.8**	**0.4±0.37**	**0.2**
Calathus ambiguus	**C**	**0.0±0.02**	**0.1**	**0.0±0.01**	**0.1**	**0.1±0.07**	**0.1**
Calathus fuscipes	O	0	-	0	-	1.2±0.44	1
Calathus melanocephalus	C	0	-	0	-	0.0±0.01	0.1
Calosoma maderae	C	0.6±0.33	0.4	0.0±0.01	0.2	0	-
Carabus violaceus fulgens	C	0	-	0	-	0.1±0.05	0.2
Clivina fossor	C	0.0±0.01	0.1	0	-	0.0±0.04	0.1
Curtonotus aulicus	P	0	-	0	-	0.0±0.02	0.1
Cylindera paludosa	C	0	-	0.1±0.04	0.2	0	-
Dixus capito	P	0.0±0.02	0.1	0	-	0.0±0.02	0.1
Egadroma marginatum	C	0.1±0.08	0.1	0	-	0	-

Table A.1. (continued) Carabid species collected by pitfall trapping in margins of maize fields (n) in three regions of NE Spain. The table shows species' mean seasonal captures (AD) ± SE and frequency (F) per trap. In bold, species common to all regions.

Species in margins (part 2)	**TG**	**Bujaraloz (n = 10)**		**Almacelles (n = 10)**		**La Seu (n = 9)**	
		AD ± SE	**F**	**AD ± SE**	**F**	**AD ± SE**	**F**
Harpalus affinis	**O**	**0.2±0.15**	**0.2**	**0.1±0.04**	**0.4**	**0.0±0.02**	**0.1**
Harpalus albanicus	P		-	0	-	0.0±0.01	0.1
Harpalus atratus	P	0.2±0.18	0.1	0	-	0	-
Harpalus distinguendus	**O**	**0.3±0.16**	**0.6**	**0.8±0.40**	**0.6**	**0.2±0.09**	**0.3**
Harpalus modestus	P	0	-	0	-	0.1±0.13	0.2
Harpalus serripes	P	0	-	0	-	0.3±0.18	0.4
Harpalus sulphuripes	P	0	-	0	-	0.0±0.13	0.1
Microlestes negrita	C	0.1±0.04	0.5	0.0±0.01	0.1	0	-
Ophonus ardosiacus	**P**	**0.0±0.03**	**0.1**	**0.1±0.09**	**0.3**	**0.0±0.01**	**0.1**
Ophonus azureus	P	0	-	0	-	0.0±0.03	0.2
Ophonus cribricollis	P	0	-	0	-	0.0±0.01	0.1
Ophonus parallelus	P	0	-	0	-	0.0±0.01	0.1
Ophonus rufibarbis	P	0	-	0	-	0.0±0.01	0.1
Pangus scaritides	P	0.1±0.06	0.1	0	-	0.0±0.03	0.2
Poecilus cupreus	**O**	**3.3±1.79**	**0.7**	**7.0±3.68**	**0.6**	**2.0±0.92**	**0.8**
Pseudoophonus rufipes	**O**	**17.9±2.72**	**1**	**8.8±2.86**	**0.9**	**4.0±1.99**	**1**
Pterostichus niger	C	0.4±0.22	0.6	0	-	0.1±0.05	0.2
Pterostichus quadrifoveolatus	C	0	-	0.0±0.01	0.1	0	-
Scybalicus oblongiusculus	P	0	-	0.1±0.08	0.1	0	-
Stomis pumicatus	C	0	-	0	-	0.0±0.01	0.1
Syntomus obscuroguttatus	C	0.1±0.07	0.5	0	-	0	-
Tachys bistriatus	C	0	-	0.0±0.01	0.1	0	-
Trechus quadristriatus	C	0.0±0.01	0.1	0	-	0	-
Zabrus tenebrioides	P	0	-	0.0±0.01	0.1	0	-

Annex 2. Carabids in alfalfa

Table A2.1. Carabid species collected by pitfall trapping in alfalfa fields (n) in three regions of NE Spain. The table shows species' mean seasonal captures (AD) ± SE and frequency (F) per trap. **In bold, species common to all regions.**

Species in alfalfa (part 1)	**TG**	**Bujaraloz (n = 6)**		**Almacelles (n = 9)**		**La Seu (n = 9)**	
		AD ± SE	**F**	**AD ± SE**	**F**	**AD ± SE**	**F**
Agonum muelleri	C	0	-	0	-	0.1 ± 0.04	0.3
Amara aenea	**O**	**0.1 ± 0.06**	**0.2**	**0.2 ± 0.18**	**0.2**	**0.1 ± 0.07**	**0.1**
Amara apricaria	P	0	-	0	-	0.0 ± 0.01	0.1
Amara montivaga	P	0	-	0.2 ± 0.13	0.4	0.0 ± 0.01	0.1
Amara subconvexa	P	0	-	0.0 ± 0.01	0.1	0.0 ± 0.01	0.1
Ancholeus nitidus	C	0.0 ± 0.02	0.2	0	-	0	-
Anchomenus dorsalis	C	0.2 ± 0.15	0.3	0.3 ± 0.17	0.3	0	-
Apotomus rufus	C	0	-	0.0 ± 0.03	0.1	0	-
Badister unipustulatus	C	0.1 ± 0.06	0.2	0	-	0	-
Bembidion ambiguum	C	0.1 ± 0.06	0.3	0.0 ± 0.03	0.2	0	-
Bembidion lampros	**C**	**0.0 ± 0.04**	**0.2**	**0.3 ± 0.16**	**0.6**	**4.3 ± 1.29**	**0.8**
Bembidion quadrimaculatum	C	0.0 ± 0.03	0.2	0.0 ± 0.03	0.2	0	-
Bembidion tethys	C	0	-	0.0 ± 0.02	0.1	0	-
Brachinus crepitans	C	0.1 ± 0.04	0.3	0	-	0.1 ± 0.06	0.1
Brachinus sclopeta	C	0.4 ± 0.27	0.5	0	-	0.2 ± 0.13	0.3
Calathus ambiguus	C	0	-	0.0 ±0.02	0.2	0.0 ± 0.04	0.1
Calathus fuscipes	O	0	-	0	-	0.9 ± 0.23	1
Calathus melanocephalus	C	0	-	0.0 ±0.02	0.1	0	-
Calosoma maderae	C	0.9 ± 0.76	0.5	0.0 ± 0.02	0.2	0	-
Clivina fossor	C	0	-	0.0 ± 0.04	0.1	0.1 ± 0.04	0.2
Cylindera paludosa	C	0	-	0.1 ± 0.07	0.2	0	-
Harpalus affinis	**O**	**0.1 ± 0.09**	**0.3**	**0.1 ± 0.06**	**0.4**	**0.1 ± 0.04**	**0.2**
Harpalus distinguendus	**O**	**0.5 ± 0.31**	**0.7**	**0.3 ± 0.19**	**0.4**	**0.1 ± 0.07**	**0.3**
Harpalus modestus	P	0	-	0	-	0.0 ± 0.01	0.1
Harpalus serripes	P	0	-	0	-	0.1 ± 0.03	0.4
Harpalus sulphuripes	P	0	-	0	-	0.0 ± 0.02	0.2
Licinus punctatulus	C	0.0 ± 0.31	0.2	0	-	0	-
Masoreus wetterhallii	C	0	-	0.0 ± 0.02	0.1	0.0 ± 0.01	0.1
Microlestes negrita	C	0.3 ± 0.19	0.5	0.2 ± 0.15	0.2	0	-
Microlestes sp.	C	0	-	0	-	0.0 ± 0.01	0.1
Myriochile melancholica	C	0.0 ± 0.03	0.2	0.0 ± 0.04	0.1	0	-
Poecilus cupreus	**O**	**4.6 ± 3.14**	**0.7**	**2.4 ± 1.06**	**0.9**	**2.3 ± 1.35**	**0.8**
Poecilus purpurascens	O	0	-	0.0 ± 0.02	0.1	0	-
Pseudoophonus rufipes	**O**	**5.6 ± 1.63**	**1**	**0.6 ± 0.21**	**0.7**	**1.3 ± 0.45**	**0.9**
Pterostichus niger	C	0.1 ± 0.08	0.3	0	-	0	-
Pterostichus quadrifoveolatus	C	0	-	0.1 ± 0.15	0.1	0	-
Syntomus obscuroguttatus	C	0.1 ± 0.09	0.3	0	-	0	-
Tachys bistriatus	C	0	-	0.3 ± 0.20	0.4	0	-
Zabrus tenebrioides	P	0.0 ± 0.02	0.2	0	-	0	-

Trophic groups (TG) are carnivore (C), omnivore (O) or phytophage (P).

Annex 3. Carabids in noncrop semi-natural vegetation

Annex 3. Carabid species collected by pitfall trapping in semi-natural vegetation (n) in three regions of NE Spain. The table shows species' mean seasonal captures (AD) ± SE and frequency (F) per trap. **In bold, species common to all regions.**

Species in semi-natural vegetation	TG	Bujaraloz (n = 9)		Almacelles (n = 5)		La Seu (n = 5)	
		AD ± SE	F	AD ± SE	F	AD ± SE	F
Agonum muelleri	C	0	-	0	-	0.0 ± 0.04	0.2
Amara consularis	O	0	-	0.0 ± 0.04	0.2	0	-
Anchomenus dorsalis	C	0	-	0	-	0.0 ± 0.02	0.2
Bembidion bipunctatum	C	0.0 ± 0.01	0.1	0	-	0	-
Brachinus crepitans	C	0.1 ± 0.12	0.1	0	-	0.2 ± 0.24	0.2
Brachinus sclopeta	C	0.1 ± 0.07	0.2	0	-	0.1 ± 0.09	0.2
Calathus ambiguus	C	0.1 ± 0.06	0.1	0	-	0.0 ± 0.03	0.4
Calathus fuscipes	O	0	-	0	-	0.3 ± 0.15	0.6
Calathus rotundicollis	C	0	-	0	-	0.3 ± 0.19	0.6
Calosoma maderae	C	0.1 ± 0.09	0.1	0	-	0	-
Carabus problematicus	C	0	-	0	-	0.0 ± 0.02	0.2
Carabus rutilans	C	0	-	0	-	0.2 ± 0.16	0.2
Dixus capito	P	0.1 ± 0.09	0.1	0	-	0	-
Harpalus affinis	O	0.0 ± 0.01	0.1	0	-	0	-
Harpalus distinguendus	O	0.0 ± 0.02	0.1	0	-	0.0 ± 0.04	0.2
Harpalus modestus	P	0	-	0	-	0.0 ± 0.02	0.2
Harpalus serripes serripes	P	0	-	0	-	0.1 ± 0.06	0.4
Laemostenus terricola	C	0.0 ± 0.01	0.1	0	-	0	-
Masoreus wetterhallii	C	0.0 ± 0.01	0.1	0	-	0	-
Microlestes negrita	C	0.0 ± 0.02	0.1	0	-	0	-
Microlestes sp1	C	0	-	0	-	0.0 ± 0.02	0.2
Microlestes sp2	C	0	-	0	-	0.0 ± 0.02	0.2
Ophonus ardosiacus	P	0	-	0	-	0.0 ± 0.02	0.2
Ophonus parallelus	P	0	-	0	-	0.0 ± 0.02	0.2
Ophonus subquadratus	P	0	-	0.0 ± 0.02	0.2	0	-
Poecilus cupreus	O	0.1 ± 0.11	0.1	0	-	0	-
Pseudoophonus rufipes	**O**	**1.0 ± 0.58**	**0.9**	**0.1 ± 0.07**	**0.2**	**0.2 ± 0.20**	**0.2**
Pterostichus niger	C	0	-	0	-	0.2 ± 0.24	0.2
Stomis pumicatus	C	0	-	0	-	0.0 ± 0.02	0.2
Syntomus obscuroguttatus	C	0.0 ± 0.02	0.2	0	-	0	-

Trophic groups (TG) are carnivore (C), omnivore (O) or phytophage (P).

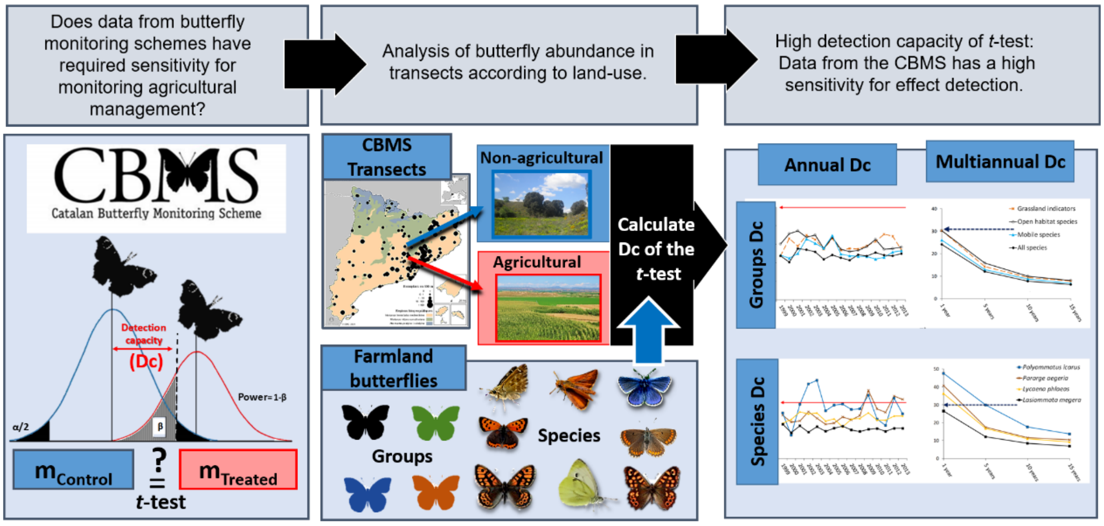
Annex 4. Sampling effort to detect carabid change in alfalfa

Table A4. Sample sizes (number of pairs of alfalfa fields) needed to detect a 30 % change in carabid populations. Calculations are based on means and standard deviation for both 2011 and 2012 combined. Data were transformed by (log (x+1)) where necessary for normalization. A pairwise t-test for independent data was used, assuming equal number of units in each group and two-tailed test, statistical power was set at 80 % (1-β = 0.8) and significance level α was 0.05.

Assessment endpoint	Bujaraloz	Almacelles	La Seu
All carabids	4	7	11
Diversity indices			
Richness (Sobs)	5	3	4
Richness (Chao 1)	5	3	4
Shannon Index	21	7	5
Trophic groups			
Omnivores	4	14	16
Carnivores	4	26	19
Phytophages	1418	337	143
Common maize species			
Pseudoophonus rufipes	9	3	51
Poecilus cupreus	36	34	42
Anchomenus dorsalis	303	288	-
Calathus fuscipes	-	-	39
Bembidion lampros	815	215	22
Calathus ambiguus	-	699	1109
Pterostichus niger	299	-	-
Harpalus serripes serripes	-	-	239
Poecilus purpurascens	-	1468	-
Harpalus distinguendus	156	222	319
Brachinus sclopeta	159	-	423
Bembidion quadrimaculatum	717	506	-
Clivina fossor	-	1109	615

Chapter 4.

The Catalan butterfly monitoring scheme has the capacity to detect effects of modifying agricultural practices



Does data from butterfly monitoring schemes have required sensitivity for monitoring agricultural management?

Analysis of butterfly abundance in transects according to land-use.

High detection capacity of *t*-test: Data from the CBMS has a high sensitivity for effect detection.

The Catalan butterfly monitoring scheme has the capacity to detect effects of modifying agricultural practices

Impacts of agricultural management practices on the receiving environment are seldom suitably assessed because environmental monitoring is costly. In this regard, data generated by already existing environmental survey networks (ESNs) may have sufficient capacity to detect effects. Here, we study the capacity of the Catalan butterfly monitoring scheme (CBMS) to detect differences in butterfly abundance due to changes in agricultural practices. As a model, we compared butterfly abundance across two landscape types according to agricultural intensification. A 2 km diameter buffer area was centred on the CBMS transect, the "control" group were transects located in areas where intensive agriculture represented less than 20% of the area; a "treated" group was simulated by selecting transects located in areas where intensive agriculture occupied an area over 40%. The Welch *t*-test (α = 0.05 and 80% power) was used to compare butterfly abundance per section across landscape types. The capacity of the *t*-test to detect changes in mean butterfly abundance, of 12 butterfly indicators relevant to farmland, was calculated annually and for 5, 10 and 15-year periods. Detection capacity of the *t*-test depended mainly on butterfly data sample size and variability; difference in butterfly abundance was less important. The *t*-test would be capable of detecting acceptably small population changes across years and sites. For instance, considering a 15-year period, it would be possible to detect a change in abundance below 10% of the multispecies indicators (all butterfly species, open habitat species, mobile species and grassland indicators); and two single species (*Lasiommata megera* and *Lycaena phlaeas*). When comparisons were carried out within each year, the *t*-test would only be capable of detecting a change below 30 % for all butterfly species, mobile species and *L. megera*. However, detection capacity rapidly improved with the addition of further years and with 5 years of monitoring, all indicators but *Thymelicus acteon* had a detection capacity below 30%. We therefore conclude that, from a statistical point of view, the CBMS data "as is" is sensitive enough for monitoring effects of changes in agricultural practices. It could be used, for instance, for the general surveillance of genetically modified crops.

Key words: Detection capacity, *t*-test, power, environmental monitoring, impact assessment, agriculture, butterfly, *Lasiommata megera*, *Lycaena phlaea*s, general surveillance, GM maize.

Changes in agricultural management practices can affect the capacity of the receiving environment to deliver ecosystem services that are essential to maintain the productivity of agricultural land (i. e. Tscharntke *et al.*, 2005). Therefore, in order to manage our resources appropriately (Ripple *et al.*, 2017), it is essential to monitor indicators capable of providing reliable information on the state of the environment before and after changes in agricultural practices (Elzinga *et al.*, 2001). However, environmental monitoring is often too costly to implement because a high number of replications in space and time are needed for reliably detecting changes of the indicators (Field *et al.*, 2007).

For this reason, there is considerable interest in using data collected by already existing survey networks (ESNs) for environmental impact assessment. The use of ESNs has received much attention (Morecroft *et al.*, 2009; Geijzendorffer & Roche, 2013); i.e. recently for monitoring the impact of GMOs on natural communities (Lang and Bühler, 2012; EFSA, 2014). However, their applicability for monitoring is still uncertain (i. e. Smets *et al.*, 2014), particularly regarding the suitability of ESN data for statistical analysis of effect detection capacity.

In order to use ESNs' data for detecting impacts, measurable effects on the receiving environment must be detected (Field *et al.*, 2007). After setting the degree of change (effect size) considered sufficient to trigger a management response, the most fundamental requirement is that the data should be capable of detecting the change if it actually occurs, that is, that it will yield adequate statistical power. For certain impacts of agricultural practices like GMOs on non-target organisms, capacity to detect population changes of 25 to 50% has been considered acceptable in field trials (Duan *et al.*, 2006; Lopez *et al.*, 2005; Perry *et al.*, 2003). Unfortunately, most ESNs lack sufficient statistical power to prevent false negative conclusions when temporal (e.g. before and after implementation of novel crop management practices) and spatial (areas where the measure has been introduced compared to areas where it has not) average differences are compared (Hails *et al.*, 2012).

Here, we carry out a case study using data from the Catalan Butterfly Monitoring Scheme (henceforth CBMS), a large-scale network based on transect counts, to determine its capacity to detect changes in mean abundance of butterfly populations due to changes in agricultural practices. This network is particularly relevant because it uses a standardised monitoring protocol for collection of butterfly data (Van Swaay *et al.*, 2008); butterflies are widely recognised ecological indicators, capable of reflecting environmental impacts of human activity in terrestrial ecosystems (Thomas, 2005); and the CBMS is located in a region where high biodiversity and agricultural intensification converge.

The main aim of this work was to determine to what extent the CBMS sampling protocol can be used "as is" for detecting a potential change, from a statistical point of view. In particular, we calculated the capacity of Welch's *t*-test (Welch, 1947) to detect eventual changes in butterfly abundance related to agricultural management.

Materials and methods

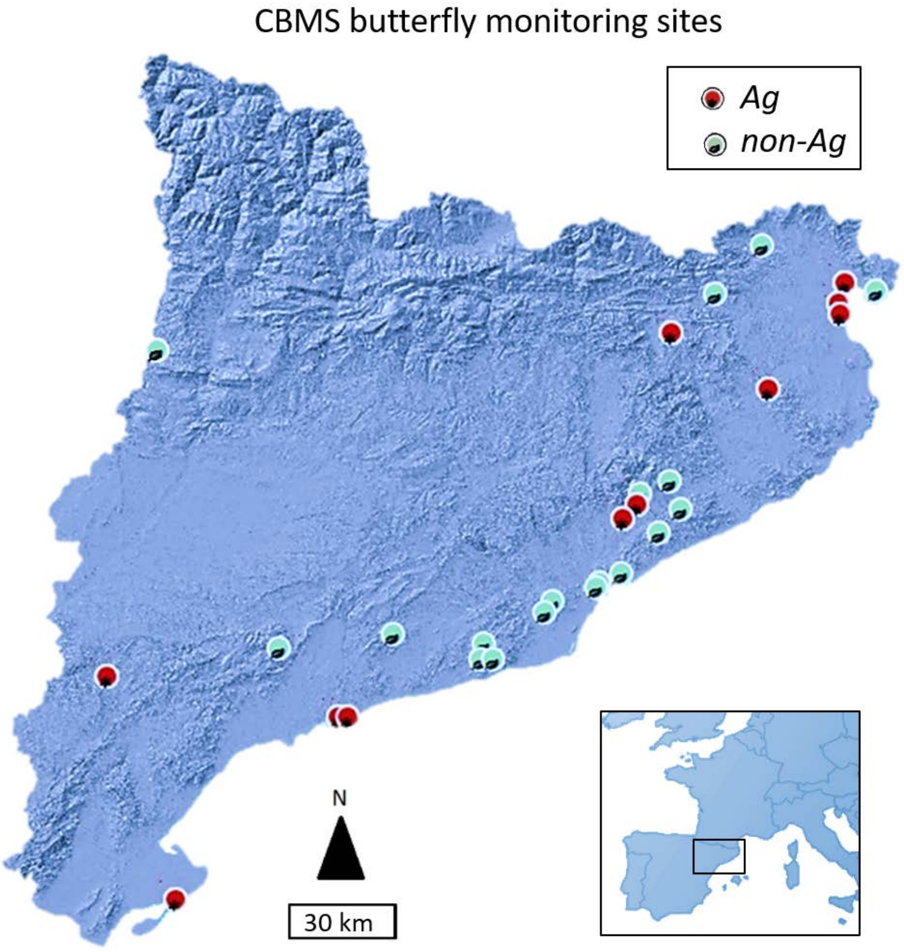
Butterfly data was provided by the CBMS (www.catalanbms.org), a network monitoring butterfly populations in Catalonia (NE Iberian Peninsula) since 1994. Butterfly data was standardised and a two means unequal variance *t*-test was used to compare abundance of four multispecies indicators and eight species, across two broad landscape types. The sensitivity of the *t*-test to detect changes in abundance between samples was calculated for two types of analysis, annual and multiannual (5, 10 and 15 years of monitoring data).

Butterfly dataset

Butterfly data was provided by the CBMS (CBMS, 2016), which currently has over 150 recording transects located throughout Catalonia, Andorra, and the Balearic Islands. The CBMS uses a standardised methodology for data collection (Pollard & Yates, 1993b), common to most European butterfly monitoring schemes (Schmucki *et al.*, 2016). In short, trained observers count the number of butterflies observed within a 5x5 m virtual area along a line transect of approximately 1.5 km, which is divided into sections of variable length according to the surrounding habitat type. Counts take place weekly from March to September, only in good weather conditions (about 30 counts per year). Transect-counts yield species-specific relative abundance indices that are assumed to reflect year-to-year population changes over the entire study area (Pollard & Yates, 1993b).

For this study, we selected a subset of the CBMS transects (Fig. 1) which were separated into two broad classes, agricultural (*Ag*) and non-agricultural (Appendix S1) (*non-Ag*), according to land cover of surrounding landscape, to test for an expected effect of land cover on butterfly communities. We would expect changes in agricultural practices to have a reduced impact on butterfly populations in *non-Ag* landscapes.Transects located in urban (more of 20% of the area covered by buildings and associated infrastructure) and montane (above 800 m) areas were excluded. Transects were also required to have been operative for at least 10 years (data was analysed from 1999 to 2013). This resulted in 11 *Ag* transects located in agricultural landscapes (i.e. where the area of arable crops and orchards was above 40% of a total area determined by a 2 km circle centred on the transect); and 18 *non-Ag* transects that were located in areas where arable crops and orchards accounted for less than 20%; *non-Ag* landscapes were dominated by grassland, scrub or forest and were often within protected areas. Land-use cover was mapped and calculated using ARCGIS version 9.3 (ESRI, 2008) on the basis of georeferenced aerial photographs (ICGC, 2013). For each butterfly indicator only sections with non-zero values were used for the analysis (i.e.Lang, 2004) as the focus of this study was on abundance data, rather than presence-absence of species (Elzinga *et al.*, 2001).

Figure 1. Location of the 29 CBMS transects analysed, grouped according to the intensity of agriculture in the surrounding landscape into agricultural (*Ag*) transects and non-agricultural (*non-Ag*) transects.



The dataset analysed comprised 135 butterfly species, many of which were present in only a few transects or years. This resulted in frequent gaps in the data making it impossible to compare populations of specific species across diverse geographical areas. To address this problem, four multispecies indicators were generated by aggregating species* according to ecological traits relevant for monitoring agricultural impacts (see suppporting information in Appendix S2 for multispecies indicator composition).

Multispecies indicators ‘all species’ included all butterfly counts (135 species); ‘open habitat species’ comprised 41 butterfly species associated to open habitats (Herrando *et al.* 2016) after excluding 12 species with a documented strong migratory behaviour, whose abundance depends heavily on the conditions at their place of origin (Stefanescu *et al.*, 2011b); ‘mobile species’ included 23 species with a high dispersal ability (Stefanescu *et al.*, 2011a); finally, ‘grassland indicators’ aggregated 16 species from the European Grassland Indicator, developed by the European Environment Agency (EEA, 2013). However, although monitoring multispecies indicators can produce very good results from a statistical point of view, the interpretation of results is more straightforward when monitoring single species. For this reason, single species potentially suitable for monitoring impacts in agricultural land were selected from the 15-year dataset. Candidate species were required to have a relative detection capacity of Welch’s *t*-test below 30% (see following sections), migrants were excluded and we selected eight single species that were widespread and common in farmland in the study area (Lee & Albajes, 2013).

Butterfly nomenclature followed Van Swaay *et al.* (2010).

*Note: in order to construct multispecies indicators, all species assigned to the same group were addedup for each sampling date and section and then calculations were performed the same as for single species.

Data analysis

Before the analysis, butterfly abundance was standarized to density (individuals/km) by dividing the sections’ butterfly abundance by section length and multiplying by 1000. Data were not transformed because for large datasets the calculated means and their deviations approximate to the normal distribution (Hazewinkel, 2002). After standardisation, mean butterfly abundance per transect section (counts from March to September) was calculated for each year (1999 to 2013). Only non-zero transect counts were used for calculations because this study focuses on the detection of change by comparing abundance only across sections where the species is present. The drawback of this method is that local extinction of species could be overlooked; nevertheless, the detection of a population decline can be used as an early warning. Standardised mean annual abundance of each butterfly indicator per section was the basic unit used in both annual and multiannual analyses.

Contrast statistic

To simulate a situation in which differences would exist between *Ag* (potentially disturbed by modified agricultural practices) and *non-Ag* landscapes (less likely exposed to disturbances by modified agricultural practices), we tested the hypothesis that there were no differences between means of sections in the two landscape types. For each butterfly indicator, the mean abundance per section in each landscape type was compared within each year (annual analysis) and also aggregating means from 5, 10 and the entire 15 year period (multiannual analysis). We used a two-sided *t*-test without assuming equal variances (Welch, 1947), a robust technique for large datasets (Fagerland, 2012) which is commonly used for field testing effects of agricultural practices (i.e. Aviron *et al.*, 2009; Feber *et al.*, 2007; Lang and Bühler, 2012). The contrast statistic (*t*) was calculated by

$$t = \frac{(m_{Ag} - m_{non-Ag})}{\sqrt{\frac{s_{Ag}^2}{n_{Ag}} + \frac{s_{non-Ag}^2}{n_{non-Ag}}}} \quad \text{[Eq. 1]}$$

where m_{Ag}, s_{Ag}, and n_{Ag}; and m_{non-Ag}, s_{non-Ag} and n_{non-Ag} are the mean, standard deviation and sample size for samples in *Ag* and *non-Ag* landscapes, respectively. The denominator is the standard error (s_e) of the statistic $m_{Ag} - m_{non-Ag}$. The significance of the test is expressed by the *p*-value (α=0.05). Correlations between longitudinal series (across years) and horizontal sampling data (between sections within a transect) were checked for lack of temporal and spatial autocorrelation.

Detection capacity of the t-test

Following Albajes *et al.* (2013), the detection capacity of the *t*-test was computed by establishing *ex ante* the probability of false positives (i.e. the probability of the test producing a significant result when there are no differences between the means of the two populations, symbolized by α, and false negatives (i.e. the probability of the test not producing a significant result, when population means are not equal, symbolized by β).

The detection capacity (*D)* of Welch's *t*-test expressed in absolute terms was computed as follows:

$$D = (t_{(1-\alpha)/2} - t_{\beta}) \times \sqrt{\frac{s_{Ag}^2}{n_{Ag}} + \frac{s_{non-Ag}^2}{n_{non-Ag}}} \quad \text{[Eq. 2]}$$

where variables were defined as in Eq 1; α was set at 0.05 and β at 0.2, values considered acceptable in field tests (Perry *et al.*, 2003), but that can be modified if required (Di Stefano, 2003; Field *et al.*, 2007). According to this procedure, the detection capacity is the size of the population change (effect size) of a given species or group that could be detected given its abundance, variability and sample size. This expression may also be used to calculate the relative detection capacity of the test in relation to the mean abundance in the control (*non-Ag* landscapes), D_N. Further details regarding this procedure can be found in Comas *et al.* (2013).

All calculations and statistical analysis were done using the R software (R Core Team, 2016); *t*-tests were carried out with R Stats Package version 3.3.1 and detection capacity calculations based on Package pwr version 1.2-1.

Results

The mean length of the 29 transects selected was 1,692 ± 132 m (mean ± SD); each transect was divided into 5 - 16 sections (mean length 198 ± 63 m). The raw dataset consisted of 262,044 butterfly section-counts, 102,210 from the 11 *Ag* transects and 159,834 from the 18 *non-Ag* transects. This is the result of considering, for each of the 135 species recorded, the number of transect sections with non-zero values in each sampling date (30 dates per year approx.), and the number of years (15). In order to compare butterfly abundance across the two landscape types, the annual mean was calculated for each section.

In the 15-year dataset, 52 species of the 135 had a relative detection capacity (D_N) below 30%; their frequency in section-counts was above 34.6% (mean sample size of 504 sections) and s_e values were generally below 1.01. From these candidates, eight indicator species widespread in farmland were selected for the annual and multiannual analyses.

Mean annual sample sizes of the multispecies indicators, 'all species' (135 species), 'open habitat species' (41 species), "grassland indicators" (16 species) or 'mobile species' (23 species) were roughly similar in both landscape types (Table 1). When the 8 single species were analysed, sample sizes were considerably lower (see Table 1 for mean annual values, the detailed year by year analysis is shown in the supporting information, Appendix S3).

When section counts were compared across 5, 10 or the entire 15-year period (multiannual analysis), sample sizes of the butterfly indicators increased greatly (Table 2) in comparison with the annual analysis (Table 1).

Table 1. Relative detection capacity (D_N) of Welch's *t*-test (α =0.05, β= 0.2) when mean butterfly abundance was compared year by year (from 1999 to 2013) across two broad landscape types.

Butterfly group/species	**Mean sample size (*n* sections)**		**Mean abundance (butterflies/km)**		**Mean SE**	**Years with significant differences**	**Mean D_N (%)**
	Ag	**non-Ag**	**Ag**	**non-Ag**			
Multispecies groups							
All species	91	115	11	14.8	1.28	13	24.1
Open habitat species	86	114	13	16.9	1.83	9	30.2
Mobile species	88	114	10	10.9	1.01	1	26
Grassland indicators	85	114	11	13.1	1.4	1	30.2
Single species							
Aricia cramera	20	40	9.5	11.1	2.32	3	59.9
Carcharodus alceae	28	27	6.7	10.3	2.49	3	68.1
Lasiommata megera	61	103	8.3	11.7	1.09	15	26.5
Lycaena phlaeas	42	55	7.7	11	1.41	11	36.2
Pararge aegeria	62	73	10	11.3	1.63	1	40.7
Pieris napi	46	22	13	9.6	1.95	5	58.3
Polyommatus icarus	68	76	14	16.6	2.92	2	47.6
Thymelicus acteon	27	45	8.9	13	3.49	0	71.6

Notes: Relative detection capacity was calculated according to Eq 2. The *Ag*: sections were located in landscapes with prevalence of agricultural habitats; *non-Ag*: ibid. but non-agricultural habitats.

Table 2. Relative detection capacity (D_N) of Welch's *t*-test (α =0.05, β= 0.2) comparing mean butterfly abundance across two broad landscape types: landscapes with prevalence of agricultural habitats (*Ag*) or with low agricultural activity (*non-Ag*). The analysis was repeated for an increasing timescale of 5 years (1999-2003), 10 years (1999-2008) and 15 years (1999- 2013).

Timespan	**Butterfly group/species**	***n***	**Dif.**	**SE**	***Sig***	**D_N (%)**
5 years	All species	445	-3.5	0.64	***	12
	Open habitat species	438	-3.8	0.96	***	15.8
	Mobile species	438	-0.7	0.49	ns	12.9
	Grassland indicators	432	-1.5	0.68	*	14.2
	Aricia cramera	140	-0.7	1.19	ns	28
	Carcharodus alceae	123	-3	0.82	***	24.4
	Lasiommata megera	366	-3.7	0.5	***	12.2
	Lycaena phlaeas	219	-3.5	0.65	***	16.7
	Pararge aegeria	315	-1.3	0.72	ns	17.5
	Pieris napi	190	2.7	0.75	***	21.8
	Polyommatus icarus	320	-3	1.89	ns	29.9
	Thymelicus acteon	152	-9.4	1.5	**	32.4
10 years	All species	1028	-3.5	0.41	***	7.8
	Open habitat species	1004	-4	0.59	***	10
	Mobile species	1013	-0.7	0.34	*	8.8
	Grassland indicators	998	-1.4	0.44	**	9.4
	Aricia cramera	315	-1	0.86	ns	20.3
	Carcharodus alceae	293	-2.7	0.6	***	17.7
	Lasiommata megera	824	-3.3	0.35	***	8.5
	Lycaena phlaeas	503	-3.4	0.43	***	11
	Pararge aegeria	706	-1	0.48	*	11.6
	Pieris napi	344	2.7	0.67	***	19.6
	Polyommatus icarus	728	-3.4	1.09	**	17.7
	Thymelicus acteon	378	-3.3	0.96	***	21.5
15 years	All species	1540	-3.5	0.34	***	6.3
	Open habitat species	1501	-4.6	0.49	***	8.1
	Mobile species	1514	-1	0.27	***	6.8
	Grassland indicators	1489	-1.6	0.36	***	7.8
	Aricia cramera	453	-1.6	0.64	*	15.9
	Carcharodus alceae	412	-3.4	0.6	***	16.7
	Lasiommata megera	1230	-3.4	0.29	***	6.9
	Lycaena phlaeas	730	-3.3	0.36	***	9.2
	Pararge aegeria	1018	-1.1	0.42	*	10.4
	Pieris napi	730	2.8	0.6	***	17
	Polyommatus icarus	1086	-2.9	0.82	***	13.7
	Thymelicus acteon	540	-3.8	0.92	***	20.1

The mean sample size (n) was the mean number of sections in each landscape type, the difference in abundance (Dif.) is butterflies/km section of Ag compared to non-Ag sections, the SE of the t-test was calculated according to Eq. 1., significant differences (Sig.) in Ag compared to the control (non-Ag) are shown by asterisks: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Factors that affected the outcome of the *t*-test

Once the significance and the power of the test have been set, the *t*-test is influenced by the difference between mean abundance of the two samples and the standard error of the test. The standard error (SE) of Welch's *t*-statistic is the square root of the sum of the ratios between the variance and the size of each sample (Eq. 1). Consequently, the larger the sample size, the smaller the SE of the test. As expected, as the average size of two samples increased, the s_e of the contrast statistic decreased exponentially ($R^2 = 0.60$):

$$s_e = 10.5 \times n^{-0.45} \quad \text{[Eq. 3]}$$

However, for similar sample sizes, the range of the test's *SE* values was quite wide, especially when considering the annual analysis (see Table 1, and Appendix S3). In multispecies data, *SE* ranged between 1.01 and 1.83 whereas average sample sizes varied in a much narrower range, 100-103, and the difference between mean abundance from 1.70 to 4.36 butterflies per km. Likewise, regarding single species, the highest *SE* values did not strictly correspond to the lowest sample sizes (Table 1).

In the multiannual analysis (Table 2), similar trends were observed. For instance, considering the entire 15-year dataset, the values of the test's *SE* differed between the four multispecies indicators (0.34, 0.49, 0.27 and 0.36 individuals per km) in spite of their relatively similar sample size (from 1,489 to 1,540). Examining single species, *SE* values were again not directly related to sample sizes. The two species with the highest *SE* (0.82 and 0.92 butterflies per km) had differing sample sizes (average 1,086 and 540); similarly, the lowest *SE* values (0.29 and 0.36 butterflies per km) also corresponded to species with relatively different sample sizes (average 1,230 and 730) (Table 2).

Detection capacity of the *t*-test

We calculated the detection capacities of Welch's *t*-test ($\alpha = 0.05$ and $\beta = 0.2$), expressed relative to the average abundance in *non-Ag* landscapes (D_N), of the four multispecies indicators and the eight single species considered. The *t*-test was carried out firstly comparing annual abundance in *non-Ag* and *Ag* landscapes year by year (Table 1 and Fig. 2A-C, detailed results in Appendix S3), and then means were compared across an increasing timespan of 5, 10 and 15-years (Table 2 and Fig. 2D-F).

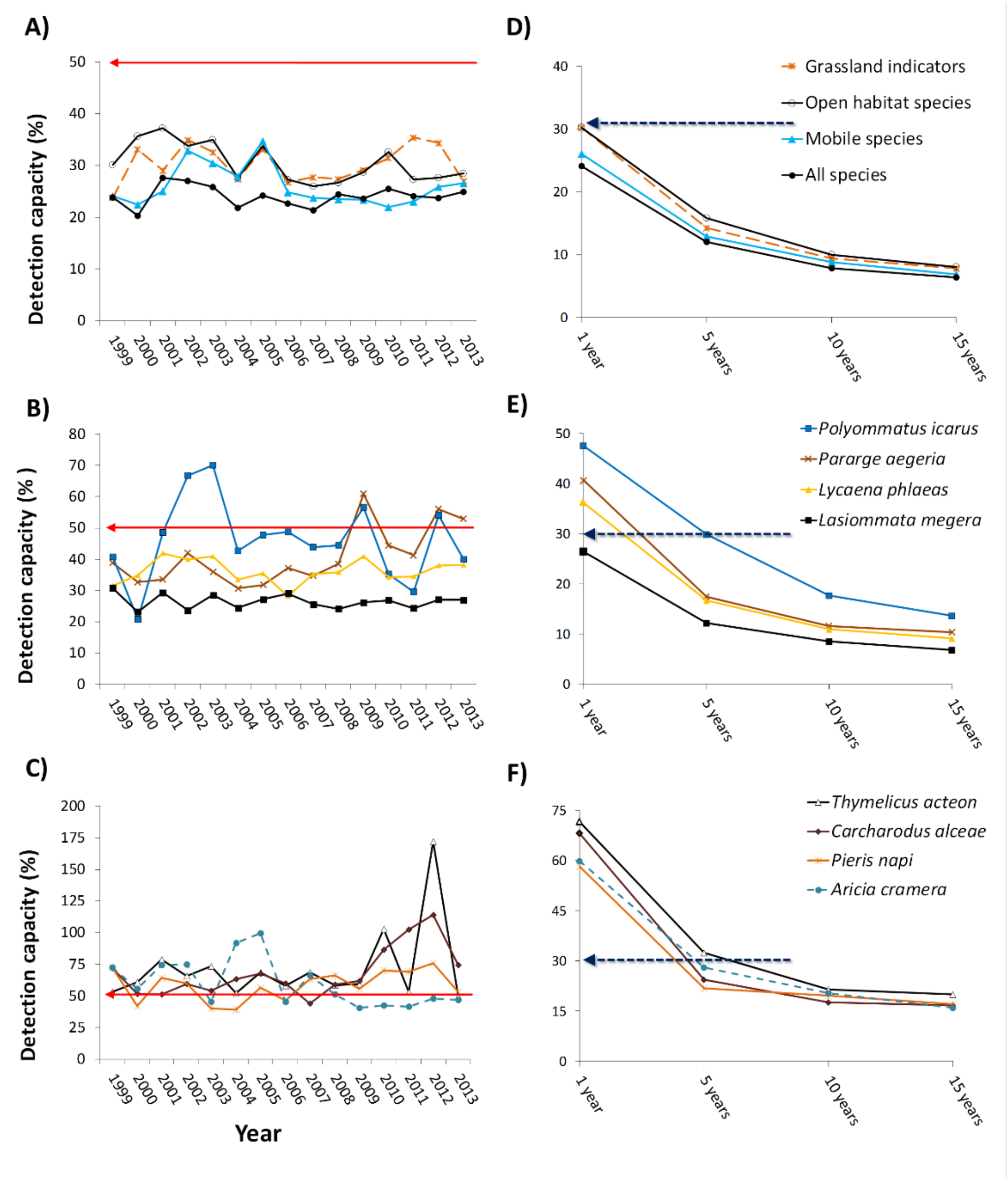


Figure 2. The figures show the relative detection capacity (D_N) of Welch's *t*-test (α = 0.05 and β = 0.2) to detect differences in butterfly abundance across two landscape use types (transects located in intensive agricultural areas compared to transects located in non-Agricultural areas). When compared within any single year (A, B, C) the *t*-test would be capable of detecting population changes below 30% of the test population (non-agricultural areas) only when butterflies are aggregated in multispecies indicators (A); when abundance of single species was compared in the two transect types within single years, D_N was only below 30 % for *Lasiommata megera* (B); for most widespread species, D_N was below 50 % (represented by the red arrow), but as sample size and abundance decreased and variability increased, the *t*-test would be unable to detect population changes below 50% of the test population (C). As data from an increasing number of years was used (5, 10 or 15 years) compared to a single year, D_N improved (a smaller population change could be detected). For the multispecies groups, in most cases two years would be sufficient to detect a 30% population change (D_N). Regarding single species, the number of years necessary for the *t*-test to detect a 30 % population difference in abundance varied; for the 7 widespread species, 5 years would already allow to detect a 30% population change (E and F); however, in the case of the less abundant and less widespread *Thymelicus acteon*, at least 6 or 7 years would be needed for the *t*-test to have the capacity to detect a change below 30 % (F). Note the different scales of the Y axis in figures.

When comparisons were conducted within each year, differences between the two landscape types were detected only in some cases (Table 1). For the four multispecies indicators, mean annual D_N values were 24.1%, 30.2%, 26.0% and 30.2% for 'all species', 'open habitat species', 'mobile species' and 'grassland indicators', respectively; D_N fluctuated always below 31% (Fig. 2A) being lowest for most years for 'all species' and then for 'mobile species'; the worst D_N values were those of 'open habitat species' and 'grassland indicators'. Regarding the 8 single species, average D_N values (Fig. 2B and 2C) ranged from 26.5% (*L. megera*) to 71.6% (*Thymelicus acteon*); only 4 species showed D_N values under 50% (Fig. 2B) in most years whereas the remaining 4 species showed very poor D_N values, consistently above 50% (Fig. 2C).

When the means comparison was carried out across an increasing time period of 5, 10, and 15-years (Table 2) the *t*-test would allow to detect increasingly small differences in population abundances between the two landscape types (see Fig. 2D-F). The effect of increasing the sample size through adding further years was very pronounced in the first 5 years and then progressively levelled off towards the end of the 15-year period.

There were differences in abundance of all the multispecies indicators and single species between landscape types, with few exceptions (Table 2). With 5 years of monitoring data, D_N values were halved for most butterfly indicators regarding the mean values of D_N obtained in the annual analysis (Fig. 2D-F) indicating a better detection capacity. When means were compared using the entire 15-year dataset, D_N dropped from a mean annual detection capacity of around 30%, to a D_N below a 10% population change, 6.3% for 'all species', 8.1% for 'open habitat species', 6.8 % for 'mobile species' and 7.8 % for 'grasland indicators' (Fig 2D). Regarding single species, the capacity of the *t*-test to detect changes in abundance between the two landscape types also improved rapidly with the addition of further years; after five years of monitoring it would be possible to detect a 30% change in abundance of all species except for *T. acteon*; after 15 years, D_N of single species (see Fig. 2E and 2F) were, on average, 13.7%, but there were considerable differences between species, ranging from 6.9% (*L. megera*) to 20.1% (*T. acteon*).

Factors influencing detection capacity

The magnitude of the test's $_{SE}$ greatly influenced both the eventual significance of the *t*-test and its ability to detect differences between population means (Eq. 2). The smaller the value of *SE*, the higher is the value of the contrast statistic, and therefore, the *t*-test is more likely to be significant. Consequently, when the value of SE is small, the test is capable of detecting very small differences between populations (low values of D), and this translates into a high detection capacity.

There was a fairly strong direct linear relationship (R^2=0.75) between the relative detection capacity (D_N) of the *t*-test and the standard error (*SE*) of the contrast statistic, described by:

$$D_N = 16.0 \times SE + 11.3 \text{ [Eq. 4]}$$

In addition, the strong dependence (R^2= 0.80) of detection capacity (D_N) on sample size (n) can be represented by an inverse potential relationship between the two variables:

$$D_N = 319.5n^{-0.51} \text{ [Eq. 5]}$$

For instance, the species with the worst D_N values, *Aricia cramera*, *Carcharodus alceae* and *T. acteon* (Figs. 2C and 2F), also had the highest *SE* values (see Tables 1 and 2, and supporting information in Appendix S3). However, there were some exceptions; for instance, considering the annual analysis, *Polyommatus icarus* had a relatively high variability but its high sample size (mean of 145 sections) and large difference between mean abundance of samples (2.76 individuals per km) resulted in an acceptable D_N; *Pieris napi*, conversely, despite a lower *SE* (average of 1.95 butterflies per km), its low sample size (mean of 68 sections) and smaller difference in mean abundance (3.00 individuals per km) resulted in a poor D_N (average of 58.3%).

Discussion

The results of this study indicate that data generated by the Catalan butterfly monitoring scheme, used “as is” has a very high sensitivity to detect impacts of modified agricultural practices on butterfly populations, provided that a suitable indicator is chosen.

Detection capacity of the CBMS data

This study shows that a *t*-test, carried out on data from a well-established butterfly monitoring scheme, would be capable of detecting an acceptably small change in butterfly abundance between two transect types, here exemplified by transects in intensive agricultural landscapes (*Ag*) compared to areas with a lower agricultural activity (*non-Ag*). When the butterfly abundance in *Ag* transects was compared to the *non-Ag* transects, using data from the entire 15-year period, the relative detection capacity (D_N)

was below 30% for 52 of the 135 species in the dataset. The species that had a good detection capacity were generally those most frequent across the landscape (translated into a large sample size) and with a relatively low variability. Considering the 12 selected butterfly indicators, D_N was below 25 % of the population abundance. This is a very good relative detection capacity, considering that population changes of 25 to 50% are considered acceptable in field trials, for instance to assess risks of GM crops on non-target organisms (Duan *et al.*, 2006; Lopez *et al.*, 2005; Perry *et al.*, 2003). Detection capacity was very good because CBMS samples were very large (> 300 sections) and the standard errors relatively moderate (< 0.95 individuals per km).

In contrast, when comparing mean butterfly abundance across the two transect types within the same year, instead of aggregating years, the values of D_N were generally much poorer (rarely below 30%) because samples were relatively small (< 30 sections) and the standard errors were relatively large (>1.9 individuals per km). For instance, four of the eight single species selected for the study (*A. cramera, C. alceae, P. napi* and *T. acteon*) may not be suitable for monitoring because the *t*-test would only be capable of detecting changes if the annual population decrease was over 50%. Conversely, it would be possible to detect a population change below 30 % of the control population of the multispecies indicators ‘all species’ and ‘mobile species’; and the single species *L. megera*, even using data from a single year. Detection capacity increased rapidly as further years were added due to a greater sample size. After 5 years, D_N was below 30% for all indicators tested except *T. acteon*. Similarly, in a recent study in which butterflies were sampled to determine the effort needed to detect a 30% reduction in abundance due to GM maize cultivation, it was found that recording 9–25 transects during 3 years would have sufficient statistical power (Lang *et al.*, 2019).

In this study, detection capacity depended heavily on sample size. This was also reported by Lang and Bühler (2012) in two Swiss butterfly monitoring schemes when mean annual abundance of multispecies indicators or single species was analysed; these authors calculated the sample size necessary for detecting changes in abundance of butterfly populations when pooled or single species’ data were used for calculations. The values describing the relationship between sample size and the detection capacity were not very different to those found in the present work except for single species in which the detection capacity was more variable than in our analysis. As Lang and Bühler (2012) only disposed of 2 datasets for some sites, they were unable to test the detection capacity using longer time-series. Worse results were obtained by Aviron *et al.* (2009) when using a dataset from a monitoring project on ecological compensation areas and biodiversity in Switzerland; this was mainly due to the low number of years (and therefore sample size) used for records. Aviron *et al.*, (2009) consequently concluded that case-specific monitoring would not be appropriate for detecting possible effects of cultivation of GM *Bt* crops on butterflies because in order to detect an effect around 30 %, over 100 pairs of fields would need to be sampled.

Regarding the butterfly indicators tested, the multispecies indicators, as expected (e.g. Brereton *et al.*, 2011; Lang and Bühler, 2012), performed much better than the single species but the drawback of using multispecies indicators is that results are difficult to interpret and effects may go unnoticed, i.e. decreases of single species can often be masked by increases of others species. This is exemplified here by the fact that there were barely any differences in abundance of ‘mobile species’ when tested annually despite significant differences for most of the single species. The most interesting species for monitoring agricultural impacts are those that are frequent across the landscape and have the lowest standard deviations, that is, those with less clumped distributions. Among these there was *L. megera,* which is a common widespread butterfly in grassland (EEA 2013) whose populations are declining across north-western Europe, possibly due to climate change (Van Dyck *et al.*, 2015). Another interesting indicator species in the study area was *Pieris napi* which, despite a poor detection capacity, was the only species more abundant in farmland. This species does not usually feed on crop plants (García-Barros *et al.*, 2013). In the arid Mediterranean climate, its presence in agricultural areas has been explained by the availability of humid environments that help to buffer the effects of extreme temperatures and droughts (Carnicer *et al.*, 2019), which are increasingly aggravated by global climate change. Finally, *T. acteon,* with its status as a Red List Species (Van Swaay *et al.*, 1999) had a rather poor relative detection capacity. This is often the case for endangered organisms, although the need to protect them from harm is very high, they are not frequent enough to allow for suitable statistical analysis.

A case study: the CBMS for general surveillance of GM crops

There are many practical cases in which it is necessary to obtain reliable information on the effects of changes in agricultural practice on the receiving environment. For instance, in the case of genetically modified (GM) crops in the EU, post market environmental monitoring (PMEM) is compulsory (Directive 2001/18/EC), and EU regulations require the implementation of general surveillance (GS) plans for long-term monitoring (EFSA, 2010). Much effort has been devoted in Europe to generate data for PMEM of GMOs, for butterflies (i.e. Lang *et al.*, 2019) and other taxa, particularly in the U.K. (Clark *et al.*, 2006) and Spain (Poza *et al.* 2005; Comas *et al.* 2014). In Spain, GM corn has been grown in thousands of hectares in the last 15 years (ISAAA, 2019), but GS has yet to be fully implemented. In order to reduce costs and increase monitoring practicability, regulation authorities recommend companies to use ESNs (EFSA GMO Panel (Panel on Genetically Modified Organisms of the European Food Safety Authority), 2011) rather than implementing field studies. There are contrasting opinions on the utility of these networks for GS of GM crops; whereas Smets *et al.* (2014) consider that these networks would only provide information on the baseline variation of indicators, other studies such as Lang and Bühler (2012) and the present study indicate that the data obtained by butterfly monitoring schemes is sensitive enough to detect changes in populations of the indicator organisms.

Whereas field studies for environmental risk assessment (ERA) of GM crops (or plant protection products) are designed specifically to detect effects, ESNs aim to obtain more general information on population dynamics. This results in differences in the factors influencing the capacity of the *t*-test to detect population change. For instance, when authors analysed data from over 12 years of field trials for ERA of GM corn, using several arthropod taxa to detect eventual changes in non-target arthropod abundance due to cultivation of *Bt* corn; taxon abundance was the most influential factor determining the relative detection capacity (D_N) of Welch's *t*-test (Albajes *et al.*, 2013). The D_N of a taxon improved as its abundance increased because its relative variability decreased. Since the number of replicates in the experimental trials was fairly constant (3-4 blocks), the standard error (SE) of the contrast statistic was determined mainly by the variability of the sample, not by sample size as in this study.

This study shows that data from the CBMS could be used to monitor post-market effects of GM crops on butterflies, given its high capacity to detect possible effects. This is a relevant point in GS of GM crops because environmental impacts of GM crops in the field, if any, would appear to be of low magnitude (Naranjo, 2009; Romeis *et al.*, 2009; Pleasants & Oberhauser, 2012; Comas *et al.*, 2014) and therefore a high detection capacity of statistical tests is needed.

Nevertheless, a drawback of the CBMS, compared to field trials, is that it mainly samples semi-natural habitats so there may be years when few transects are located in the vicinity of GM, at the beginning of *Bt* corn deployment; and non-GM corn fields, when *Bt* corn has been successfully established in the area. This would result in a reduction of detection capacity. To address this issue, EFSA (2014) recommended the possible increase of transect number, which would also increase costs (Schmeller & Henle, 2008). Nevertheless, this problem should be mitigated in future because butterfly monitoring schemes are attempting to increase number and spatial uniformity of monitoring sites in agricultural landscapes, where butterfly populations may suffer more pressures from agricultural practices (Brereton *et al.*, 2010). Additionally, the increase in number of sites could also be partially compensated by a lower sampling frequency (Brereton *et al.*, 2010). Notwithstanding the pitfalls of ESN data, is often not possible to use field trials due to the costs involved, so data from these monitoring schemes represent the only practicable option for environmental monitoring.

Conclusion: is the CBMS suitable for monitoring?

With probability levels fixed at α=0.05 and β=0.2, a Welch's *t*-test on CBMS data had the capacity to detect changes in abundance between 6% and 20% of the selected butterfly indicators when samples were compared across a 15-year period. Detection capacity was found to depend mainly on sample size, thus species that were more frequent across the landscape tended to have higher detection capacities. When mean butterfly abundances between transects were compared within the same year, the sensitivity of the *t*-test was much lower but it would still be possible to detect population changes between 24% and 50 % in 8 out of the 12 butterfly indicators tested. Detection capacity rapidly improved with the addition of further years as this greatly increased sample size, and after 5 years of monitoring it would be possible to detect differences in abundance below a 30% threshold. In conclusion, this study shows that, despite some pitfalls, data from existing environmental survey networks do have the potential to be used for environmental monitoring of agrienvironmental measures to inform shareholders and policymakers. In the specific case of GM crops, the CBMS could be used to monitor post-market effects on butterflies, given its high capacity to detect possible effects. However, the specific testing approach would have to be adapted to the nature of the expected agricultural impacts to be monitored and to the particular characteristics and limitations of the monitoring scheme data.

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Supplementary material Chapter 4

Appendix S1. CBMS Transects

Table S1. Characteristics of the 29 CBMS sampling sites (linear transects) analysed in the study.

Transect type	CBMS site	Arable land (% area)	Start date	UTM	Transect length (m)	Sections (n)	Section length ± SD (m)	Mean annual Temp. (ºC)	Mean annual rainfall (mm)	Elevation (m asl)
Agricultural (*Ag*)	1	76	1988	31T EG07	4296	16	269 ± 29.0	15	628	2
	9	47	1994	31T DG56	1672	13	129 ± 16.0	12	1100	539
	10	58	1994	31T DG41	1664	9	185 ± 42.9	14	791	334
	23	75	1997	31T EG08	1100	6	183 ± 21.9	15	651	1
	29	64	1997	31T DG41	2030	9	226 ± 70.5	14	720	265
	38	60	1999	31T CF55	1850	9	206 ± 31.5	16	537	205
	48	71	2001	31T BF96	1265	9	141 ± 23.6	16	371	58
	52	76	2001	31T CF65	2476	7	354 ± 44.4	16	549	5
	59	83	2002	31T EG07	1266	5	253 ± 83.2	15	616	1
	67	55	2002	31T CF10	1919	8	240 ± 58.6	17	526	3
	77	42	2004	31T DG84	1770	13	136 ± 16.1	14	813	138
Non-agricultural (*non-Ag*)	5	11	1994	31T DG89	1695	12	141 ± 14.9	14	853	175
	8	1	1994	31T DF28	2642	14	209 ± 14.9	15	708	239
	11	0	1994	31T DG42	2083	10	208 ± 31.0	12	869	772
	13	11	1994	31T DG61	2298	9	255 ± 36.5	15	825	258
	19	12	1995	31T DG52	1719	9	191 ± 23.0	13	843	537
	21	9	1996	31T DF28	840	5	168 ± 27.4	15	693	331
	26	1	1997	31T DF07	1049	8	131 ± 15.4	14	684	317
	33	7	1999	31T DG50	846	9	94 ± 15.0	13	846	486
	34	9	1999	31T DF39	791	6	132 ± 28.8	15	654	136
	36	2	1999	31T DF07	1452	5	290 ± 77.0	14	673	319
	45	0	2000	31T DF07	799	6	133 ± 24.3	15	641	176
	51	11	2001	31T CF47	1626	7	232 ± 30.7	13	676	598
	58	19	2001	31T CF77	1092	9	121 ± 18.3	14	672	121
	64	2	2002	31T EG17	1283	8	160 ± 29.9	15	656	27
	66	13	2003	31T CG06	1743	7	249 ± 36.2	12	738	536
	68	11	2002	31T DF39	2102	7	300 ± 48.0	14	696	327
	69	8	2003	31T DF49	2030	9	226 ± 22.6	14	712	406
	70	15	2003	31T DG77	1662	10	166 ± 27.8	14	919	248

Transects were assigned to two categories according to the intensity of the agricultural activity in the surrounding landscape; in the 11 agricultural (Ag) transects, more than 40% of the area in a 2 km buffer zone was occupied by arable crops and orchards whereas the 18 non-agricultural (non-Ag) transects were located in landscapes where intensive agriculture represented less than 20% of the total area. Further information regarding transects can be found at www.catalanbms.org

Appendix S2. Multispecies indicator composition

Table S2. Butterfly species included in the multispecies indicators tested for assessing environmental effects of agricultural measures: “mobile species” aggregated 23 species with high dispersion capacity in the study areas (Stefanescu *et al.*, 2011a) the “open habitat species” aggregated 41 species linked to open habitats in the area (from Herrando *et al.* 2016).

Mobile species

Aglais io (Linnaeus, 1758)
Aglais urticae (Linnaeus, 1758)
Anthocharis cardamines (Linnaeus, 1758)
Argynnis pandora (Dennis & Schiffermüller, 1775)
Cacyreus marshalli (Butler, 1898)
Carcharodus alceae (Esper, 1780)
Celastrina argiolus (Linnaeus, 1758)
Charaxes jasius (Linnaeus, 1767)
Euchloe crameri (Butler, 1869)
Gonepteryx cleopatra (Linnaeus, 1767)
Gonepteryx rhamni (Linnaeus, 1758)
Hipparchia semele (Linnaeus, 1758)
Iphiclides feisthamelii (Duponchel, 1832)
Issoria lathonia (Linnaeus, 1758)
Lasiommata megera (Linnaeus, 1767)
Libythea celtis (Laicharting, 1782)
Lycaena phlaeas (Linnaeus, 1761)
Nymphalis antiopa (Linnaeus, 1758)
Nymphalis polychloros (Linnaeus, 1758)
Pararge aegeria (Linnaeus, 1758)
Pieris napi (Linnaeus, 1758)
Polygonia c-album (Linnaeus, 1758)
Polyommatus icarus (Rottemburg, 1775)

Open habitat species

Aglais urticae (Linnaeus, 1758)
Anthocharis euphenoides (Staudinger, 1869)
Aporia crataegi (Linnaeus, 1758)
Argynnis adippe (Dennis & Schiffermüller, 1775)
Argynnis aglaja (Linnaeus, 1758)
Aricia cramera (Eschscholtz, 1821)
Boloria dia (Linnaeus, 1767)
Brintesia circe (Fabricius, 1775)
Coenonympha arcania (Linnaeus, 1761)
Coenonympha pamphilus (Linnaeus, 1758)
Colias alfacariensis (Ribbe, 1905)
Cupido alcetas (Hoffmannsegg, 1804)
Cupido argiades (Pallas, 1771)
Erynnis tages (Linnaeus, 1758)
Euchloe crameri (Butler, 1869)
Euphydryas aurinia (Rottemburg, 1775)
Glaucopsyche alexis (Poda, 1761)
Gonepteryx cleopatra (Linnaeus, 1767)
Gonepteryx rhamni (Linnaeus, 1758)
Hipparchia semele (Linnaeus, 1758)
Hipparchia statilinus (Hufnagel, 1766)
Iphiclides feisthamelii (Duponchel, 1832)
Issoria lathonia (Linnaeus, 1758)
Leptidea sinapis (Linnaeus, 1758)
Lycaena phlaeas (Linnaeus, 1761)
Maniola jurtina (Linnaeus, 1758)
Melanargia lachesis (Hübner, 1790)
Melitaea deione (Geyer, 1832)
Melitaea didyma (Esper, 1778)
Melitaea phoebe (Dennis & Schiffermüller, 1775)
Polyommatus bellargus (Rottemburg, 1775)
Polyommatus coridon (Poda, 1761)
Polyommatus escheri (Hübner, 1823)
Polyommatus icarus (Rottemburg, 1775)
Pseudophilotes panoptes (Hübner, 1813)
Pyrgus malvoides (Elwes & Edwards, 1897)
Pyronia cecilia (Vallantin, 1894)
Pyronia tithonus (Linnaeus, 1767)
Satyrium esculi (Hübner, 1804)
Thymelicus acteon (Rottemburg, 1775)
Thymelicus sylvestris (Poda, 1761)

Table S2 (continued). Butterfly species included in the multispecies indicators tested for assessing environmental effects of agricultural measures: the "grassland indicators" aggregated 16 indicator species for European grasslands, an indicator developed by the European Environmental Agency (EEA, 2013); finally the list of 12 migrant species that were excluded from the groups because their abundance depends heavily on the conditions at their place of origin (Stefanescu *et al.*, 2011b).

Grassland indicators	
Anthocharis cardamines (Linnaeus, 1758)	*Maniola jurtina* (Linnaeus, 1758)
Coenonympha pamphilus (Linnaeus, 1758)	*Ochlodes sylvanus* (Esper, 1777)
Cupido minimus (Fuessly, 1775)	*Phengaris arion* (Linnaeus, 1758)
Cyaniris semiargus (Rottemburg, 1775)	*Polyommatus bellargus* (Rottemburg, 1775)
Erynnis tages (Linnaeus, 1758)	*Polyommatus coridon* (Poda, 1761)
Euphydryas aurinia (Rottemburg, 1775)	*Polyommatus icarus* (Rottemburg, 1775)
Lasiommata megera (Linnaeus, 1767)	*Spialia sertorius* (Hoffmannsegg, 1804)
Lycaena phlaeas (Linnaeus, 1761)	*Thymelicus acteon* (Rottemburg, 1775)
Migratory species	
Colias crocea (Geoffroy, 1785)	*Papilio machaon* (Linnaeus, 1758)
Danaus chrysippus (Linnaeus, 1758)	*Pieris brassicae* (Linnaeus, 1758)
Danaus plexippus (Linnaeus, 1758)	*Pieris rapae* (Linnaeus, 1758)
Gegenes nostrodamus (Fabricius, 1793)	*Pontia daplidice* (Linnaeus, 1758)
Lampides boeticus (Linnaeus, 1767)	*Vanessa atalanta* (Linnaeus, 1758)
Leptotes pirithous (Linnaeus, 1767)	*Vanessa cardui* (Linnaeus, 1758)

Appendix S3. Annual analysis of differences in butterfly abundance

Table S3.1. Annual differences between butterfly abundance (considering multispecies indicators) of CBMS transects located in areas of high agricultural intensity (*Ag*) regarding transects located in areas of low agricultural activity (*non-Ag*). Comparisons were carried out for each year (1999 to 2013) using a two sample unequal variance *t*-test (α =0.05, β= 0.2). significant differences are shown by different letters following mean abundance (butterflies/km) in each landscape type. The relative detection capacity (D_N) of the test was calculated by Eq 2. as percent population change (regarding *non-Ag*) that the test could could detect.

Indicator group or species	**Year**	**Sample size (*n* sections)**		**Abundance (butterflies/km)**		**Se of *t*-test**	***p*-value**	**D_N (%)**
		Ag	**non-Ag**	**Ag**	**non-Ag**			
All species combined	**1999**	**62**	**85**	**11.1 b**	**13.5 a**	**1.1**	**0.038**	**24**
	2000	**61**	**81**	**11.3 b**	**13.9 a**	**1.2**	**0.034**	**20**
	2001	75	109	11.5 a	14.3 a	1.4	0.051	28
	2002	**84**	**98**	**11.4 b**	**15.3 a**	**1.5**	**0.008**	**27**
	2003	**91**	**143**	**11.7 b**	**16.7 a**	**1.5**	**0.001**	**26**
	2004	**102**	**143**	**11.8 b**	**17.1 a**	**1.3**	**0.000**	**22**
	2005	**95**	**141**	**11.1 b**	**13.5 a**	**1.2**	**0.037**	**24**
	2006	**104**	**135**	**11.4 b**	**14.9 a**	**1.2**	**0.003**	**23**
	2007	**107**	**124**	**10.8 b**	**14.5 a**	**1.1**	**0.001**	**21**
	2008	104	112	10.8 a	12.7 a	1.1	0.095	24
	2009	**104**	**115**	**12.4 b**	**15.5 a**	**1.3**	**0.018**	**24**
	2010	**101**	**114**	**11.3 b**	**16.1 a**	**1.5**	**0.001**	**26**
	2011	**95**	**112**	**10.7 b**	**14.4 a**	**1.2**	**0.004**	**24**
	2012	**87**	**109**	**10.8 b**	**14.9 a**	**1.3**	**0.002**	**24**
	2013	**86**	**100**	**12.1 b**	**14.7 a**	**1.3**	**0.042**	**25**
Open habitat species	1999	60	85	13.1 a	15.5 a	1.7	0.142	30
	2000	58	81	14.2 a	14.8 a	1.9	0.733	32
	2001	72	109	13.3 a	15.8 a	2.1	0.235	37
	2002	83	98	13.0 a	16.9 a	2.0	0.061	34
	2003	**86**	**143**	**13.8 b**	**21.2 a**	**2.6**	**0.005**	**35**
	2004	**98**	**142**	**13.4 b**	**21.2 a**	**2.1**	**0.000**	**28**
	2005	91	139	12.5 a	13.7 a	1.6	0.477	34
	2006	**102**	**132**	**11.6 b**	**15.9 a**	**1.5**	**0.006**	**27**
	2007	**99**	**122**	**11.7 b**	**17.6 a**	**1.6**	**0.000**	**26**
	2008	96	111	11.7 a	13.3 a	1.3	0.191	27
	2009	**96**	**115**	**12.1 b**	**18.4 a**	**1.9**	**0.001**	**29**
	2010	**95**	**114**	**12.4 b**	**19.7 a**	**2.3**	**0.002**	**33**
	2011	**91**	**112**	**11.7 b**	**17.0 a**	**1.6**	**0.001**	**27**
	2012	**80**	**109**	**11.4 b**	**16.3 a**	**1.6**	**0.003**	**28**
	2013	**83**	**100**	**12.0 b**	**16.1 a**	**1.6**	**0.013**	**29**

Table S3.1. (continued). Annual differences between butterfly abundance (considering multispecies indicators) of CBMS transects located in areas of high agricultural intensity (*Ag*) regarding transects located in areas of low agricultural activity (*non-Ag*). Comparisons were carried out for each year (1999 to 2013) using a two sample unequal variance *t*-test (α =0.05, β= 0.2). significant differences are shown by different letters following mean abundance (butterflies/km) in each landscape type. The relative detection capacity (D_N) of the test was calculated by Eq 2. as the percent population change (regarding *non-Ag*) that the test would be capable of detecting.

Indicator group or species	Year	Sample size (*n* sections)		Abundance (butterflies/km)				SE of *t*-test	*p*-value	D_N (%)
		Ag	non-Ag	Ag		non-Ag				
Mobile species	1999	60	84	9.7	a	9.9	a	0.8	0.803	24
	2000	59	81	9.7	a	10.4	a	0.8	0.401	23
	2001	73	107	9.6	a	10.8	a	1.0	0.247	25
	2002	82	98	10.4	a	11.8	a	1.4	0.320	33
	2003	89	143	10.4	a	10.6	a	1.2	0.874	30
	2004	98	142	10.4	a	11.4	a	1.1	0.350	28
	2005	93	141	10.4	a	10.0	a	1.2	0.743	35
	2006	102	134	10.3	a	10.9	a	1.0	0.510	25
	2007	104	124	9.9	a	10.7	a	0.9	0.364	24
	2008	100	112	10.1	a	12.1	a	1.0	0.052	23
	2009	98	115	9.7	a	11.0	a	0.9	0.172	23
	2010	**96**	**114**	**9.4**	**b**	**11.2**	**a**	**0.9**	**0.047**	**22**
	2011	93	111	10.4	a	10.4	a	0.8	0.246	23
	2012	84	108	9.7	a	10.8	a	1.0	0.300	26
	2013	83	100	9.8	a	11.9	a	1.1	0.060	27
Grassland indicators	1999	60	84	10.9	a	12.6	a	1.1	0.125	24
	2000	57	81	12.5	a	12.8	a	1.5	0.829	33
	2001	69	108	11.6	a	13.0	a	1.3	0.295	29
	2002	79	98	12.2	a	14.9	a	1.8	0.134	35
	2003	88	140	11.9	a	13.3	a	1.5	0.383	33
	2004	96	142	11.9	a	13.2	a	1.3	0.317	27
	2005	92	140	11.3	a	11.8	a	1.4	0.740	33
	2006	101	132	11.4	a	13.6	a	1.3	0.098	27
	2007	99	121	10.7	a	12.5	a	1.2	0.138	28
	2008	97	112	12.0	a	12.7	a	1.2	0.587	27
	2009	92	114	11.6	a	12.4	a	1.3	0.528	29
	2010	93	114	11.3	a	13.7	a	1.5	0.116	31
	2011	91	110	10	a	13.3	a	1.7	0.057	35
	2012	79	109	11	a	12.5	a	1.5	0.342	34
	2013	**80**	**100**	**10.9**	**b**	**13.7**	**a**	**1.3**	**0.037**	**27**

Table S3.2.1. Annual differences between butterfly abundance (considering single species) of CBMS transects located in areas of high agricultural intensity (*Ag*) regarding transects located in areas of low agricultural activity (*non-Ag*). Comparisons were carried out for each year (1999 to 2013) using a two sample unequal variance *t*-test (α =0.05, β= 0.2). significant differences are shown by different letters following mean abundance (butterflies/km) in each landscape type. The relative detection capacity (D_N) of the test was calculated by Eq 2. as the percent population change (regarding *non-Ag*) that the test would be capable of detecting.

Indicator group or species	**Year**	**Sample size (*n* sections)**		**Abundance (butterflies/km)**		**SE of *t*-test**	***p*-value**	**D_N (%)**
		Ag	**non-Ag**	**Ag**	**non-Ag**			
Aricia cramera	1999	18	16	11.3 a	9.0 a	2.2	0.304	72
	2000	19	23	10.5 a	11.0 a	2.1	0.795	59
	2001	18	50	11.6 a	11.7 a	3.0	0.990	74
	2002	20	47	13.5 a	13.3 a	3.4	0.962	75
	2003	21	47	9.6 a	12.5 a	2.0	0.159	46
	2004	11	44	8.9 a	13.7 a	4.4	0.280	92
	2005	19	42	12.1 a	9.4 a	3.2	0.409	99
	2006	26	48	10.6 a	13.0 a	2.1	0.257	46
	2007	26	48	11.0 a	11.7 a	2.7	0.788	66
	2008	37	49	9.8 a	11.0 a	2.0	0.544	51
	2009	28	45	8.0 a	9.8 a	1.4	0.198	41
	2010	**20**	**45**	**6.9 b**	**10.9 a**	**1.6**	**0.016**	**43**
	2011	18	37	6.9 a	9.4 a	1.4	0.067	41
	2012	**11**	**23**	**6.3 b**	**10.3 a**	**1.7**	**0.025**	**48**
	2013	**15**	**34**	**6.1 b**	**10.1 a**	**1.6**	**0.020**	**47**
Carcharodus alceae	1999	17	29	8.1 a	9.0 a	2.2	0.671	72
	2000	**18**	**19**	**5.7 b**	**9.1 a**	**1.6**	**0.046**	**52**
	2001	20	29	7.0 a	8.9 a	1.6	0.231	51
	2002	23	31	5.9 a	9.6 a	2.0	0.074	59
	2003	**31**	**29**	**5.9 b**	**10.4 a**	**1.9**	**0.028**	**54**
	2004	29	38	7.5 a	9.3 a	2.1	0.373	63
	2005	34	28	6.1 a	8.1 a	1.9	0.302	68
	2006	33	37	8.0 a	10.2 a	2.2	0.304	60
	2007	**33**	**43**	**6.0 b**	**10.5 a**	**1.6**	**0.007**	**44**
	2008	35	29	7.6 a	9.1 a	1.9	0.428	59
	2009	27	18	5.9 a	10.1 a	2.2	0.069	62
	2010	27	26	7.3 a	11.2 a	3.3	0.252	86
	2011	37	23	6.5 a	12.9 a	4.5	0.172	102
	2012	23	13	7.0 a	15.0 a	5.6	0.183	114
	2013	28	16	5.7 a	10.6 a	2.7	0.086	75

Table S3.2.2. Annual differences between butterfly abundance (considering single species) of CBMS transects located in areas of high agricultural intensity (*Ag*) regarding transects located in areas of low agricultural activity (*non-Ag*). Comparisons were carried out for each year (1999 to 2013) using a two sample unequal variance *t*-test (α =0.05, β= 0.2). significant differences are shown by different letters following mean abundance (butterflies/km) in each landscape type. The relative detection capacity (D_N) of the test was calculated by Eq 2. as the percent population change (regarding *non-Ag*) that the test would be capable of detecting.

Indicator group or species	Year	Sample size (*n* sections)		Abundance (butterflies/km)		SE of *t*-test	*p*-value	D_N (%)
		Ag	non-Ag	Ag	non-Ag			
Lasiommata megera	1999	45	77	7.9 b	11.1 a	1.2	0.010	31
	2000	50	81	7.5 b	11.2 a	0.9	0.000	23
	2001	58	98	8.5 b	11.3 a	1.2	0.017	29
	2002	59	92	8.7 b	13.3 a	1.1	0.000	24
	2003	66	106	6.8 b	10.9 a	1.1	0.000	29
	2004	57	134	8.8 b	11.8 a	1.0	0.004	24
	2005	55	120	7.8 b	10.3 a	1.0	0.013	27
	2006	78	123	9.8 b	12.4 a	1.3	0.042	29
	2007	68	112	8.0 b	11.9 a	1.1	0.000	26
	2008	66	102	8.7 b	12.3 a	1.1	0.001	24
	2009	60	104	7.6 b	11.4 a	1.1	0.000	26
	2010	61	110	8.7 b	12.9 a	1.2	0.001	27
	2011	67	99	7.9 b	10.8 a	0.9	0.003	24
	2012	54	96	7.8 b	11.5 a	1.1	0.001	27
	2013	65	97	9.3 b	11.7 a	1.1	0.032	27
Lycaena phlaeas	1999	39	52	7.9 a	9.9 a	1.1	0.077	32
	2000	34	42	7.3 b	11.3 a	1.4	0.005	35
	2001	28	65	7.5 b	10.7 a	1.6	0.046	42
	2002	37	56	7.0 b	12.0 a	1.7	0.004	40
	2003	27	57	7.1 b	10.5 a	1.5	0.026	41
	2004	49	69	7.6 b	11.3 a	1.3	0.006	33
	2005	58	67	7.8 a	9.7 a	1.2	0.114	35
	2006	49	76	8.1 b	12.1 a	1.2	0.001	28
	2007	48	52	7.3 b	10.1 a	1.3	0.025	35
	2008	45	56	8.3 b	12.2 a	1.5	0.013	36
	2009	42	39	8.7 a	11.3 a	1.6	0.118	41
	2010	46	49	8.2 a	10.5 a	1.3	0.072	34
	2011	52	49	7.4 a	9.3 a	1.1	0.094	34
	2012	38	51	8.1 b	12.9 a	1.7	0.007	38
	2013	37	50	7.5 b	11.4 a	1.5	0.013	38

Table S3.2.3. Annual differences between butterfly abundance (considering single species) of CBMS transects located in areas of high agricultural intensity (*Ag*) regarding transects located in areas of low agricultural activity (*non-Ag*). Comparisons were carried out for each year (1999 to 2013) using a two sample unequal variance *t*-test (α =0.05, β= 0.2). significant differences are shown by different letters following mean abundance (butterflies/km) in each landscape type. The relative detection capacity (D_N) of the test was calculated by Eq 2. as the percent population change (regarding *non-Ag*) that the test would be capable of detecting.

Indicator group or species	Year	Sample size (*n* sections)		Abundance (butterflies/km)		SE of *t*-test	*p*-value	D_N (%)
		Ag	non-Ag	Ag	non-Ag			
Pararge aegeria	1999	50	61	10.0 a	9.9 a	1.4	0.902	39
	2000	49	54	10.2 a	10.8 a	1.2	0.592	33
	2001	49	63	9.1 a	11.2 a	1.3	0.124	34
	2002	58	76	11.2 a	11.5 a	1.7	0.878	42
	2003	64	106	10.8 a	13.4 a	1.7	0.133	36
	2004	69	90	10.3 a	10.5 a	1.1	0.830	31
	2005	61	85	10.0 a	9.5 a	1.1	0.619	32
	2006	71	93	11.9 a	11.5 a	1.5	0.760	37
	2007	79	96	10.9 a	12.3 a	1.5	0.365	35
	2008	71	67	9.8 a	13.4 a	1.8	0.055	39
	2009	63	59	9.7 a	12.6 a	2.7	0.297	61
	2010	63	63	9.2 a	9.9 a	1.5	0.645	44
	2011	71	72	11.0 a	11.4 a	1.7	0.801	41
	2012	56	54	9.7 a	10.4 a	2.1	0.722	56
	2013	61	61	10.0 a	11.3 a	2.1	0.114	53
Pieris napi	**1999**	**27**	**13**	**14.4 a**	**8.8 b**	**2.2**	**0.025**	**72**
	2000	30	16	11.6 a	10.8 a	1.6	0.619	42
	2001	**45**	**17**	**13.5 a**	**7.1 b**	**1.6**	**0.000**	**64**
	2002	40	24	12.1 a	11.0 a	2.3	0.650	60
	2003	48	27	11.8 a	10.0 a	1.4	0.232	40
	2004	73	42	12.1 a	9.7 a	1.3	0.105	39
	2005	41	27	11.9 a	10.0 a	2.0	0.383	56
	2006	64	25	13.0 a	9.6 a	1.6	0.062	46
	2007	**57**	**24**	**13.4 a**	**8.5 b**	**1.9**	**0.017**	**63**
	2008	42	21	12.2 a	9.6 a	2.2	0.270	66
	2009	40	21	11.9 a	8.8 a	1.7	0.082	56
	2010	58	26	13.0 a	15.4 a	3.8	0.544	70
	2011	49	17	12.1 a	8.3 a	2.0	0.089	69
	2012	**33**	**11**	**14.2 a**	**8.3 b**	**2.2**	**0.011**	**76**
	2013	**44**	**16**	**12.2 a**	**8.6 b**	**1.6**	**0.035**	**53**

Table S3.2.4. Annual differences between butterfly abundance (considering single species) of CBMS transects located in areas of high agricultural intensity (*Ag*) regarding transects located in areas of low agricultural activity (*non-Ag*). Comparisons were carried out for each year (1999 to 2013) using a two sample unequal variance *t*-test (α =0.05, β= 0.2). significant differences are shown by different letters following mean abundance (butterflies/km) in each landscape type. The relative detection capacity (D_N) of the test was calculated by Eq 2. as the percent population change (regarding *non-Ag*) that the test would be capable of detecting.

Indicator group or species	Year	Sample size (*n* sections)		Abundance (butterflies/km)		SE of *t*-test	*p*-value	D_N (%)
		Ag	non-Ag	Ag	non-Ag			
Polyommatus icarus	1999	50	54	13.3 a	14.8 a	2.1	0.493	41
	2000	46	44	16.4 a	13.6 a	3.3	0.403	44
	2001	59	66	13.8 a	18.9 a	3.2	0.121	49
	2002	70	76	13.5 a	22.5 a	5.3	0.091	67
	2003	74	100	17.0 a	16.9 a	4.2	0.995	70
	2004	76	106	13.2 a	16.8 a	2.5	0.166	43
	2005	71	90	11.1 a	12.2 a	2.1	0.609	48
	2006	82	85	11.9 a	14.9 a	2.6	0.244	49
	2007	74	71	13.2 a	16.4 a	2.5	0.213	44
	2008	**77**	**81**	**16.2 b**	**24.6 a**	**3.9**	**0.031**	**44**
	2009	77	76	14.6 a	15.2 a	3.0	0.844	57
	2010	70	85	13.1 a	15.2 a	1.9	0.273	35
	2011	69	80	10.1 a	11.9 a	1.3	0.147	30
	2012	62	59	17.3 a	14.7 a	2.8	0.371	54
	2013	**64**	**74**	**13.5 b**	**20.8 a**	**2.9**	**0.014**	**40**
Thymelicus acteon	1999	11	33	7.8 a	11.7 a	2.1	0.082	53
	2000	10	32	8.6 a	10.3 a	2.1	0.432	62
	2001	26	39	7.7 a	12.3 a	3.4	0.183	79
	2002	18	53	9.3 a	13.2 a	3.1	0.203	66
	2003	23	59	9.3 a	15.5 a	4.0	0.127	73
	2004	38	88	9.8 a	13.4 a	2.5	0.156	52
	2005	27	49	7.6 a	11.8 a	2.8	0.149	68
	2006	46	51	10.8 a	12.5 a	2.5	0.508	58
	2007	38	49	9.1 a	11.8 a	2.8	0.341	68
	2008	26	39	9.5 a	10.3 a	2.1	0.711	58
	2009	25	37	7.5 a	10.9 a	2.3	0.134	59
	2010	39	48	10.3 a	15.5 a	5.6	0.353	103
	2011	31	39	8.7 a	10.4 a	1.9	0.398	53
	2012	24	21	7.6 a	21.8 a	12.8	0.276	172
	2013	28	33	9.4 a	13.1 a	2.3	0.114	50

Addendum to Appendix S3. Annual abundance of butterflies and population trends in Ag and non-Ag landscape types.

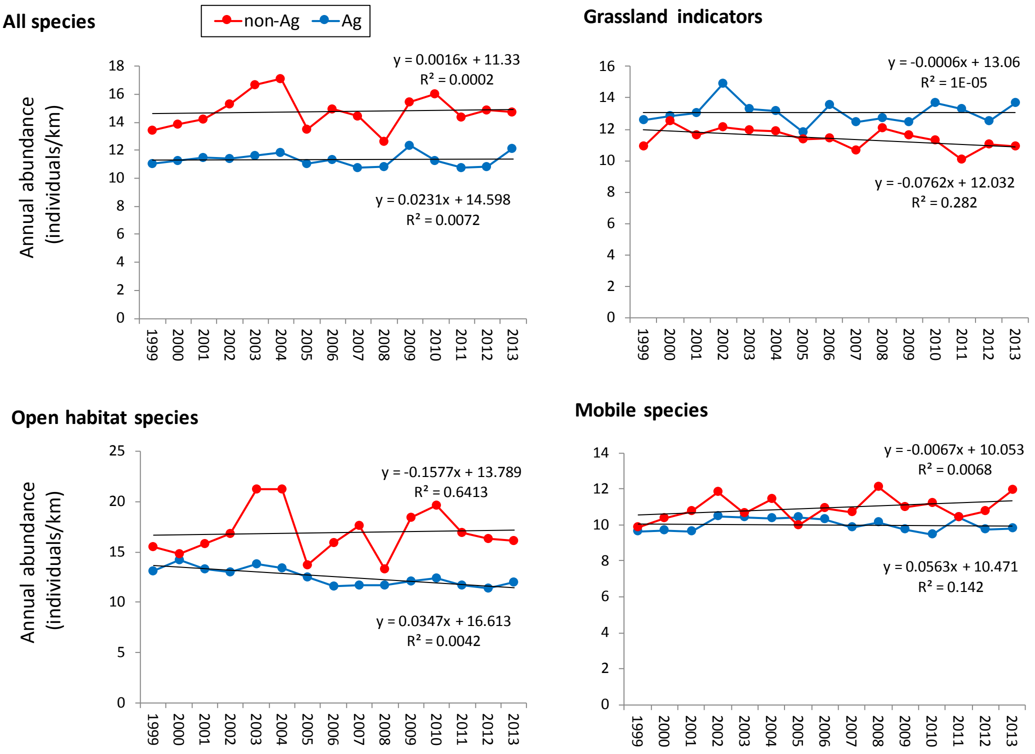


Fig. S3.1. Annual abundance and population trends of multispecies butterfly indicators in CBMS transects located in areas of high agricultural intensity (*Ag*) and transects located in areas of low agricultural activity (*non-Ag*) for the period between 1999 to 2013.

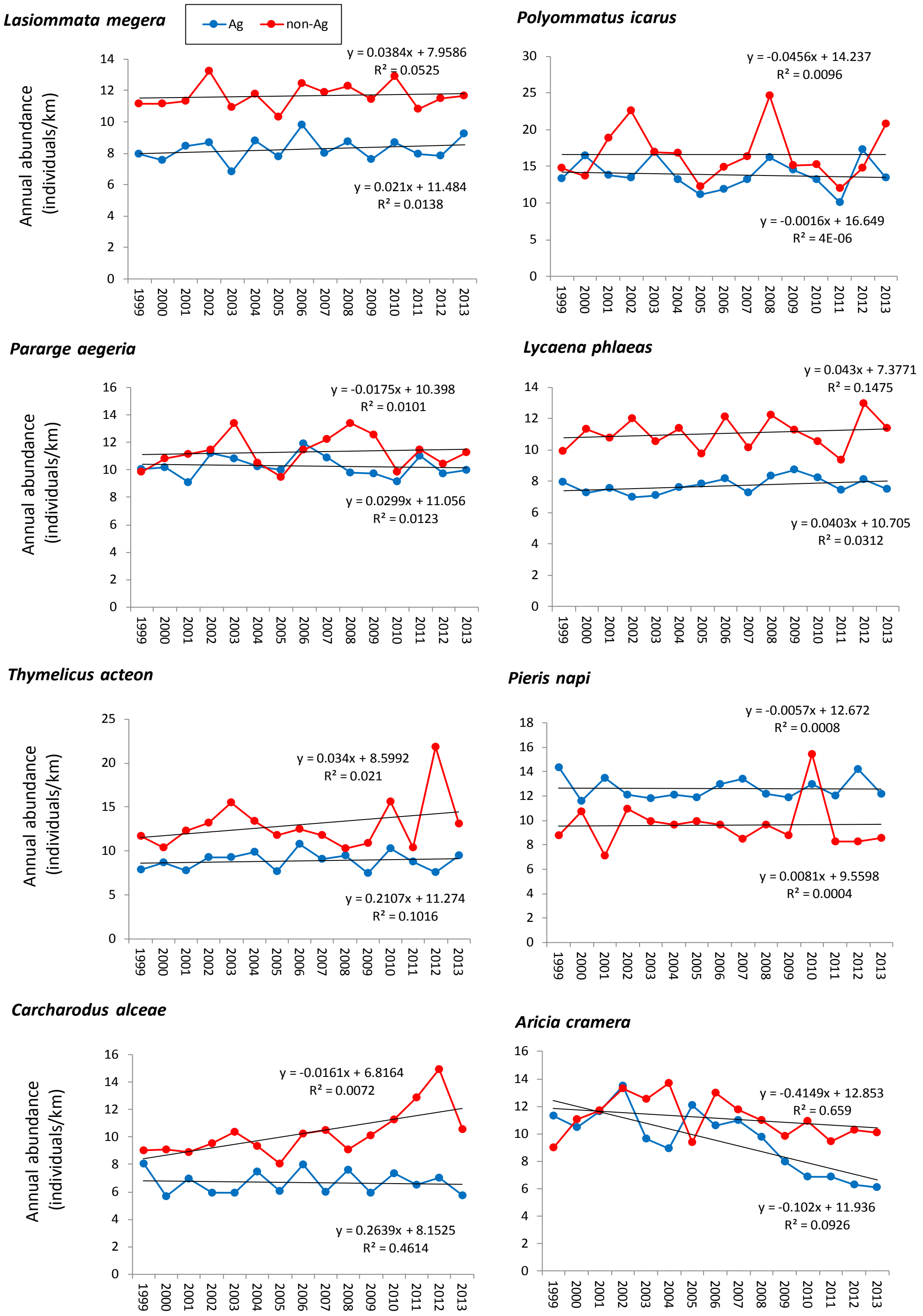
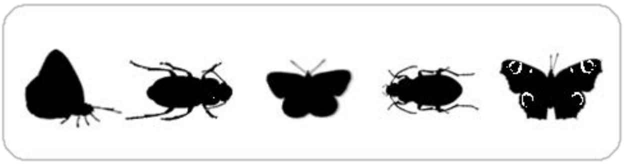


Fig. S3.2. Annual abundance and population trends of butterfly species in CBMS transects located in areas of high agricultural intensity (*Ag*) and transects located in areas of low agricultural activity (*non-Ag*) for the period between 1999 to 2013.

General discussion



General discussion

This thesis was carried out in order to identify and address issues required to increase the feasibility of General Surveillance of GM maize in the EU, particularly in Spain where this crop is widely cultivated. Chapters 1 and 2 dealed with the identification and selection of appropriate butterfly indicators for monitoring GM maize impacts in the field. Chapter 3 dealed with the identification and selection of carabid indicators. Finally, Chapter 4 focused on the capacity of the Catalan Butterfly Monitoring Scheme to detect differences in abundance of certain butterfly indicators.

An environmental monitoring plan using butterflies

In the field study carried out in three maize growing regions in NE Spain, butterflies turned out to be far more abundant and diverse in maize agroecosystems than expected beforehand. In fact, three European red list species were detected throughout the study. Butterflies were abundant in the three habitats sampled across the different regions.

Lycaenid butterflies were very abundant in alfalfa because it is a larval host plant to many species of that family, moroever when alfalfa was in flower butterflies of many different species flocked to the flowers for the nectar.

Field margins were usually the habitat where butterflies were most abundant because this is a transition area where the floral diversity is higher than in the crops and there were often more flowers than in the neighbouring vegetation (Pywell *et al.*, 2004). This finding was in agreement with other authors focusing on butterfies in agricultural systems; for instance, Wallis de Vries *et al.*, (2017) also found that maize field margins harboured high butterfly abundance in the Netherlands. This finding is particularly relevant for GS of GM maizes because it highlights the fact that there are a number of butterflies that are exposed to impacts derived from changes in management of maize and other agricultural crops. However, the importance of field margins in sustaining butterfly populations in agricultural areas requires further study in order to understand how changes in agricutural management might impact the overall butterfly populations.

As part of the thesis, butterflies in noncrop seminatural vegetation were surveyed also because *Bt* maize pollen can drift onto host plants in these areas (Lang *et al.*, 2015). Even in the peak of summer when temperatures can reach 40ºC, there were butterflies present in the arid landscapes of the Ebro basin, in many cases the species observed in the noncrop areas were dryland specialists such as *Pyronia cecilia* that were almost never found in field margins or alfalfa. This finding highlights the fact that despite intensive agriculture, the remaining fragments of natural dryland vegetation still harbour a considerable biological richness and diversity (this finding is also supported by the flora surveys conducted in the noncrop seminatural vegetation).

Among the butterfly species that were common around maize anthesis in the study regions, a considerable number of them appear suitable for effect assessment of GM maizes according to the indicator selection criteria considered. This study provides a rather comprehensive pool of butterfly species from which indicator species could be selected for GS of GM maize. It is certainly noteworthy that the most suitable species varied broadly from one region to another, indicating the importance of performing a field survey in each of the receiving environments in order to identify the most suitable species for monitoring and to design a suitable monitoring plan for each diferentiated maize-growing region. In this regard, a floral survey is very strongly recommended prior to the implementation of the GS plan because it can be used to assess the potential exposure of the different butterfly species. The first steps towards the design of a GS plan for butterflies is to take into account which species may be most exposed, and in this regard, ideally, larvae should be sampled. Nevertheless, in this study very few larvae were found considering the sampling effort involved; therefore, it would not appear suitable to include larval sampling of most species as part of the GS plan. Despite this, it could be feasible to monitor certain larval species in some conditions, specifically, we found that sampling *Carcharodus alceae* on mallow, *Malva* spp. could be feasible in the Lleida region. In other regions, *C. alceae* were not as abundant as in Lleida so this option could be less feasible.

Field monitoring of butterfly indicators using the design described in Chapters 1 and 2 would appear feasible for GS, particularly if multispecies indicators are used, such as the European Grassland Indicator (EEA, 2013). According to the estimated sampling effort, it would theoretically be possible to detect relatively small butterfly population changes (30%) between sites where GM maize is cultivated compared to a comparator (sites where non-GM is cultivated). Our results contrast to those found by Aviron *et al.*, (2009) who found that in Switzerland case-specific field monitoring of butterflies would require an excessive sampling effort.

It is clear from our results that butterfly monitoring would have to use paired sites in the same maize-growing region because there were large differences in the identity and frequency of butterflies and their host plants between the different maize-growing regions that we surveyed in this study, similar to differences observed at wider biogeographic scales (Dolezel *et al.*, 2018a; Lang *et al.*, 2019).

The sampling methodology that we used in this study was not very intensive, a 300 m linear transect at each site was sampled three times per season. Nevertheless, using this design it would still be possible to detect changes in abundance of some single species using only 22 site pairs, and multispecies indicators using only19 site pairs. A reduction in the number of sampling sites could be achieved by increasing transect length or number of visits as this would probably allow to reduce the number of sampling sites (Comas *et al.*, 2015; Lang *et al.*, 2016). For instance, in a recent study estimating the required sample size and monitoring effort necessary to run a Lepidoptera survey in European farmland, we found that a 30% decrease in butterfly abundance could be detected using 9–25 transects and only 4 visits, providing that transect length was over 800 m (Lang *et al.*, 2019); the estimated sampling effort of such a monitoring plan was equivalent to around 20 working days per year, which would appear fully feasible.

Moroever, the most feasible option for GS of GM maizes to determine potential effects on butterfly indicators would be the integration of field monitoring of GM maize with the use of data from ESNs such as the Catalan Butterfly Monitoring Scheme (CBMS). This would allow to reduce the number of sampling sites specifically used for monitoring GM maizes. Nevertheless, it would still be necessary to maintain some sites in close contact to GM maize and non-GM maize in order to determine the causality of observed effects, that is if GM maize cultivation has differential effects compared to non-GM maize.

An environmental monitoring plan using carabids

Butterflies are well known and valued by the general public because of their beauty. In addition, they carry out important ecosystem functions as pollinators and they are part of the trophic web as primary consumers of plants and as prey to predators and parasitoids. However, in the maize agroecosystem their contribution to crop productivity may be rather low. In this regard, carabids can be considered much more valuable to the productivity and sustainability of agricultural ecosystems because they contribute to biological control of weed seeds and crop pests (Kromp, 1999). Moroever, they are abundant within the maize fields and their larvae develop belowground, where they are in very close and prolonged contact with the maize crop and its residues, as well as herbicide residues. Therefore, from the agronomical point of view and from the point of view of exposure, carabids are highly relevant organisms for monitoring effects of GM crops in the field. The main drawback of using carabids is the fact that there are currently no ESNs that routinely monitor their populations and therefore a GS plan using carabids as indicator organisms would necessarily require the design and implementation of field surveys. Anyhow, the design and implementation of a generalised scheme to assess the effects of agrienvironmental measures on biodiversity in agricultural land is long overdue.

We lack tools and information to reliably assess the effects of different agrienvironmental measures both at the local and the landscape scales. The productivity and sustainability of our agricultural land depends in great measure on the different organisms that form part of the biocenosis and carry out essential ecological functions such as nutrient recycling, biological control of weeds and pests or pollination. For instance, in the EU, the Common Agricultural Policy (CAP) is increasingly focused on protecting the environment and the agricultural resources; currently most payments to farmers are conditioned to the implementation of environmental measures on their farms (EC, 2020). Despite this, the effects of these environmental measures on biodiversity and ecological function are seldom reliably assessed (EU, 2020). Environmental monitoring is the only way to reliably assess the effects of these measures both at the local and community levels; and reliable effect assessment requires relevant and sensitive indicators that can be monitored using cost-effective methods. In this regard, carabids are highly relevant to agricultural ecosystems, they are sensitive to environmental change and they can be cost-effectively sampled using pitfall traps (Luff, 1996). Thus, it would appear feasible to include carabids into a general surveillance plan for effect assessment of agrienvironmental measures in general, and for GM maize in particular.

In this study, carabid assemblages, similarly to butterflies, varied between study regions although there were some species that were ubiquitous. Such was the case of the omnivorous seed-feeder *Pseudoophonus rufipes*. In Chapter 3 this species was recomended for GS of GM maize because it was the most sensitive for effect detection from a statistical point of view, it is easy to trap and identify and it fulfils a valuable role as a weed seed predator. Nevertheless, similarly as recomended for butterflies, different carabid indicators could be used in differentiated maize-growing regions.

In contrast to butterfly sampling, carabids were also sampled within maize fields. Butterflies are rarely observed within the maize fields because there are few larval plants or flowers. In contrast, carabid larvae develop in the soil of maize fields and adults are found quite frequently in arable fields (e.g. Holland & Luff, 2000). Thus, most carabids are liable to be more exposed to onsite impacts derived from cultivation of GM maizes, particularly due to flora- or prey-mediated effects. Changes in carabid communities could have negative effects on crop productivity through the impairment of the biological control function (Kromp, 1999).

Carabids were clearly most abundant in field margins, as they probably use this habitat for shelter, for seeking food or for moving between other habitats (e.g. Holland & Luff, 2000b; Thomas *et al.*, 2001; Madeira & Pons, 2015) therefore, in Chapter 3 we recomended to sample field margins. This is also one of the preferred sites for butterfly monitoring because of the abundance of larval host plants and nectar-rich flowers and therefore both carabids and butterflies could be sampled in the same field margins.

The sampling effort required to detect a 30 % reduction in carabid abundance was much lower than in the case of butterflies and sampling only 3 to 24 site pairs three times per growing season would already allow to detect a 30% reduction in abundance of *P. rufipes*. The low sampling effort required to detect effects on carabid populations coupled to their relevance and value in the agrienvironment make them excellent candidates for GS to detect effects of GM maize within a general environmental monitoring to assess the effects of agrienvironmental measures in general.

Data from the CBMS can be used for General Surveillance

According to the results found in the field studies, carabids would appear to be the best option for field monitoring but the use of butterfly indicators have a very clear advantage that cannot be matched by any other arthropod group: butterflies are highly valued by the public. Their attractiveness and ease of sampling have led to the involvement of many volunteers who contribute butterfly data free of charge. This has allowed the development of widespread butterfly monitoring schemes that have continuity in space and time thanks to the commitment of the volunteers. Data from these butterfly monitoring schemes have been used to understand the effects of changes in landscape, in climate and in agricultural practices (e.g. Stefanescu *et al.*, 2011; Espeset *et al.*, 2016; Mills *et al.*, 2017) and they also appear to be highly suitable for effect detection of GM maizes according to our study and others (e.g. Lang & Bühler, 2012; Lang *et al.*, 2019).

The majority of butterfly monitoring schemes across the EU share a similar methodology (Schmucki *et al.*, 2016) and there is an increasing number of recording sites in agricultural land (e.g. Brereton *et al.*, 2011) even if it means reducing the number of visits. In this regard, (Lang *et al.*, 2016) found that reducing the recording frequency from seven to four inspections per season still yielded 80–90 % of the species, as long as peak abundances in summer months were included.

In Chapter 4, the butterfly abundance data obtained by the Catalan Butterfly Monitoring Scheme was analysed in order to determine if it could be used for environmental monitoring of changes in agricultural practices. The capacity of a robust *t*-test (Welch *t*-test) to detect differences in butterfly abundance between agricultural sites and a comparator was very high. In other words, data from the CBMS can be used to reliably detect very small differences in abundance of farmland butterfly indicators (below 10% when 15-year datasets were used). We therefore concluded that, from a statistical point of view, the CBMS data was sensitive enough for monitoring effects of changes in agricultural practices such as GM maize cultivation.

According to the study of CBMS data, the species with the greatest capacity to reflect population changes were *L. megera*, *L. phlaeas*, *P. aegeria* and *P. icarus*; other single species (for instance *T. acteon*, *C. alceae* or *P. napi*) would only be sufficiently sensitive if multiyear (at least 5 years) data was used. When we sampled the three maize agroecosystems, we found that *P. icarus* was also widespread and abundant in the maize agroecosystem. *Pieris. napi* appeared as the second single species that was widespread and abundant enough to allow a feasible monitoring effort across the three regions.

It is worth mentioning that in this study, sections were used as replicates because we found that this greatly increased the power of the tests and correlations were rare. Nevertheless, this aspect should be taken into account and checked when designing a monitoring plan because the more mobile species could move across sections in the same site and also there could be site-specific differences in butterfly dynamics that could influence results.

Another aspect that would have to be considered in order to design a sound General Surveillance plan is the number of sites located in areas where the GM crop is cultivated compared to the non GM comparator. Data from the CBMS can be used for GS of GM maize providing a sufficient number of transects are established in areas where GM maize is cultivated and non-GM comparator.

According to our field study (Chapter 2) there are considerable differences in butterfly population composition and abundance even at a reduced biogeographical scale. Any General Surveillance plan using data from monitoring schemes that cover heterogeneous areas must take into account these differences by carrying out field studies in maize agroecosystems to identify the most appropriate indicators.

Which have been the effects of *Bt* maize cultivation?

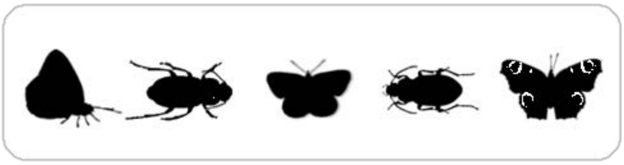
Bt maize MON810 has been cultivated for almost 15 years in the Ebro basin but we have no idea about how the butterfly communities in maize agroecosystems may have changed over time. However, it is clear that the deployment of *Bt* maize has not resulted in a complete loss of butterfly populations. It is even possible that Lepidoptera in general may have benefitted from the reduction in insecticide treatments that used to be commonplace to control maize borers and that have now been almost completely substituted by deployment of *Bt* maize. This could be the case because many field studies have found that unsprayed *Bt* maize benefitted arthropod biodiversity compared to non-GM maize sprayed with insecticides (Bhatti *et al.*, 2006; Dively, 2006; Eckert *et al.*, 2006; Rose & Dively, 2007; Naranjo, 2009). However, there is no reliable information regarding how the deployment of MON810 has affected butterfly populations in the cultivation areas. As mentioned previously, this situation is not restricted to GM crops. In general, there is a considerable lack of knowledge on how adoption of different agrienvironmental management options affect biodiversity because to date there are very few monitoring schemes focusing on how management affects biodiversity in agricultural areas.

Outlining an improved General Surveillance plan

In summary, a GS plan including field monitoring of butterflies and carabids could provide reliable data on the effects of GM maize cultivation on biodiversity and ecological functions of non-target arthropods; the use of data from butterfly monitoring schemes is an extremely powerful tool to assess potential effects of cultivation of GM crops at a wider scale.

The field monitoring of GM maizes outlined in this thesis could be integrated into a widescale environmental monitoring to assess effects of agrienvironmental measures, as advocated by other authors and EU institutions (Wilhelm *et al.*, 2009; EFSA, 2014). In the case of butterflies, the farm-scale or regional scale results regarding butterflies could be better understood using data from butterfly monitoring schemes that provide standardised, reliable data across large areas of the EU (Schmucki *et al.*, 2016), integrated with landscape analysis (Leclerc *et al.*, 2018). Such a GS plan would require some fine-tuning and it would be highly desirable to include additional indicators such as soil organisms involved in nutrient recycling (van Capelle *et al.*, 2016). Nevertheless, the indicators and methods presented in this thesis could greatly increase the effect detection capacity of the GS plan for GM maize that is currently implemented in the EU.

Conclusions



Conclusions

The overall conclusion of this thesis is that General Surveillance of Genetically Modified maizes could be substantially improved through field sampling of butterflies and carabids. Moroever, data from butterfly monitoring schemes would allow to greatly increase the effect detection capacity of the General Surveillance plan implemented for MON810. Some guidelines for improvement of the design of the monitoring plan are provided.

Monitoring butterfly indicators in the field

1. Butterflies are abundant and frequent across different habitats in the maize agroecosystem.
2. Larval host plants of butterflies are frequent in maize fields, maize field margins, other crops and noncrop vegetation.
3. Selection of appropriate butterfly indicators for GS of GM maize depends on the maize-growing region.
4. In the conditions of this study, sampling butterfly larvae as part of the GS plan would probably require excessive effort but it could be feasible for certain plants and butterfly species, for instance *Carcharodus alceae* on *Malva* spp.
5. Field monitoring of butterflies for GS of GM maize requires a high sampling effort but it could be feasible for multispecies groups or single species such as *Pieris napi* or *Polyommatus icarus.*

Monitoring carabid indicators in the field

1. Carabids are abundant and frequent across different habitats in the maize agroecosystem.
2. The best carabid indicator was *Pseudoophonus rufipes*, satisfying criteria of abundance, relevance, sensitivity, ease of sampling, and sufficient statistical power.
3. The carnivore trophic group should also be monitored as an indicator of biodiversity and biological control of invertebrates.

4. The best location for sampling carabids was the field margin where carabids are exposed to GM maize and are abundant enough to require the smallest sample sizes to detect population changes.

Can ESNs be used for GS of GM maize?

1. The Catalan butterfly monitoring scheme (CBMS) was identified as the most suitable existing environmental surveillance network (ESN) in the study area.
2. The detection capacity of the *t*-test depended mainly on butterfly data sample size and variability; difference in butterfly abundance was less important.
3. The *t*-test would be capable of detecting acceptably small changes in butterfly abundance across years and sites for GS of GM maize.
    a. When comparisons were carried out within each year, the *t*-test would only be capable of detecting a change below 30 % two multispecies groups and the single species *Lasiommata megera*.
    b. Detection capacity rapidly improved with the addition of further years and with 5 years of monitoring, almost all butterfly indicators tested have a detection capacity below 30%.
4. Data generated by ESN has the required sensitivity for monitoring impacts of GM crop, nevertheless, the methodology must be adapted to make sure that the butterflies monitored are in close contact with the GM crops and their non-GM counterparts.

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