Project Number 289706

COLLABORATIVE PROJECT

AMIGA

Assessing and Monitoring the Impacts of Genetically modified plants on Agro-ecosystems

D7.2 Adaptation of methodologies for Lepidoptera GMO monitoring and validation on AMIGA field sites (M1-M48)

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Pictures on front page (© Andreas Lang)

Left: Scarce Swallowtail (*Iphiclides podalirius*), Mureni, Romania.

Middle: Butterfly monitoring alongside a maize field on an AMIGA transect in Apold, Romania.

Right: Crepuscular Burnet (*Zygaena carniolica*), Mureni, Romania.

Summary

The specific objectives of AMIGA Task 7.2 “Adaptation of methodologies for Lepidoptera GMO monitoring and validation on AMIGA field sites” were (i) development, testing and validation of a standardised monitoring approach for a GMO monitoring of Lepidoptera, (ii) identification of a baseline of the occurring number of lepidopteran species and their abundance in farmland in representative regions of Europe, (iii) information about variance of both species richness and abundance of day-active Lepidoptera in farmland, and (iv) estimation of the involved costs for a farmland butterfly monitoring scheme.

A field protocol was developed specifying the implementation of a GMO butterfly monitoring with the following set-up: linear transect routes (“Pollard walks”) to be installed along the field (margins), transect length of 1 km, the transects to be walked away and back, four inspections per season (May, June, July, August), and censuses to be repeated yearly. In addition, relevant environmental variables must be recorded such as crop and habitat type, flower intensity in field margins, or weather conditions during the actual monitoring.

The generated butterfly monitoring protocol was tested and validated in the field in three maize growing regions of representative bio-geographical zones of Europe, i.e., Transylvania/Romania, Catalonia/Spain and Scania/Sweden. The surveys based on these protocols were carried out during three seasons from 2013 to 2015. Transects were placed in field margins, and all the day-active Lepidoptera (Papilionoidea, Hesperiidae, Zygaenidae) were monitored according to the specified protocol. The suggested monitoring protocol proved to be an adequate and reliable method to monitor farmland butterflies and burnet moths. The recorded baseline revealed a relatively high biodiversity of butterflies in farmland of regions in Romania and Spain, including rare and protected species, and a lower species richness in Sweden. The coefficient of variation (CV) for species number was different between countries being lowest in Sweden and highest in Spain. Within countries, the CV was relatively stable in different years for 1 km long transects. Transects shorter than 1 km reported fewer species, and showed a high and unstable CV.

Statistical power calculations showed that, in a given region, the typical sample size to detect a 10 % loss of Lepidoptera species is 15 – 30 transects, and about 20 transects to detect a 30 % decrease in their total abundance. However, unanticipated events and outliers can increase necessary sample size by a factor of 2 to 10, therefore, a precautionary sample size of 30 to 60 transects per region appears sensible. Required sample size for analysing specific subgroups of species is slightly higher, e.g., for common species (+15 %), red list species (+30 %), or EEA grassland butterfly indicator species (+60%).

Further, the actual time spent in the field for the monitoring of farmland Lepidoptera was recorded. Following the developed monitoring design, the effort would generally range between 11 to 40 working days per year in a given region (not including organisational and administrative tasks). The monitoring effort would increase if accounting for unexpected outliers and analyses for specific butterfly species groups.

Designing an effective monitoring scheme for Lepidoptera requires important decisions of how to design the monitoring scheme, and how to allocate the available resources and efforts. The results of deliverable 7.2 demonstrate that a cost-efficient monitoring to detect advers effects on farmland butterflies is realistic and feasible. The deliverable 7.2 provides significant guidelines for predictions of power and cost-efficiency of future Lepidoptera GMO monitoring programmes to be implemented in European farmland.

Contents

1. Background

In the European Community, the Directive 2001/18/EC on the Deliberate Release into the Environment of Genetically Modified Organisms (GMOs) stipulates a monitoring plan in order to trace and identify any harmful effects on human health or the environment of GMOs after they have been placed on the market (EC 2001). Guidelines with regard to the requirements for monitoring design, sampling methods and analysis techniques are outlined in further documents of the European Community (EC 2002, EFSA 2011). The Directive 2001/18/EC distinguishes two parts of post-market environmental monitoring (PMEM): general surveillance and case-specific monitoring (EC 2001). Case-specific monitoring should, when included in the monitoring plan, focus on potential adverse effects of GMOs that have been identified in the previous environmental risk assessment (ERA). Thus, a case-specific monitoring plan would serve to confirm or reject the assumptions of the ERA, and case-specific monitoring should address specific hypotheses associated with identified potential effects of the GM crop (EC 2002). In contrast, general surveillance should focus on unanticipated and unforeseen as well as on possible delayed and long-term effects that were not predicted in the risk assessment. If unexpected changes in the environment have been observed, further risk assessment may need to be considered to establish whether they have arisen as a consequence of GMO cultivation (EC 2002). General surveillance should, where compatible, make use of established routine surveillance practices such as ecological monitoring, environmental observation and nature conservation programmes (EC 2002, EFSA 2014).

Butterflies and moths were often suggested as relevant parameters to be recorded in a GMO monitoring plan (e.g. Lang 2004; Sanvido et al. 2004). In general, Lepidoptera are considered sensitive and valuable bio-indicators, because they can indicate various states and changes in the environment such as conditions of climate, vegetation, habitat or the landscape (Aviron et al. 2007a; Settele et al. 2009; but see Fleishman and Murphy 2009 for a critical evaluation of the use of Lepidoptera as indicators). This includes the assessment of agri-environmental schemes (Aviron et al. 2007b, Roth et al. 2008), the detection of effects on biodiversity (Wenzel et al. 2006, Nilsson et al. 2008), the recording of management effects in arable land (Field et al. 2005, 2007, Dover et al. 2010) or adverse effects of pesticide use (e.g. Johnson et al. 1995, Russell and Schultz 2010), and the impact of land use change (e.g. Stefanescu et al. 2009, van Dyck et al. 2009). The features contributing to the value of Lepidoptera as environmental indicators further include the good knowledge on their faunistics, ecology and conservation biology, relatively easy identification of species and the presence of field guides, existence of sound and widely accepted monitoring methods, the establishment of many volunteer monitoring schemes in Europe and the wider public acceptance of Lepidoptera as valuable protection goals (Skinner 1998, Bachellard et al. 2007, VanSwaay et al. 2008, Settele et al. 2009). In addition, Lepidoptera fulfil important ecological key roles as herbivores, pollinators and prey organisms in many terrestrial ecosystems. Depending on the specific circumstances, butterflies can be representative for general biodiversity, and potentially indicate changes in other animal groups and plants (Thomas 2005, Thomas et al. 2004). Recently, the dramatic decline of grassland butterflies in Europe caused strong concern (Van Swaay et al. 2015).

Currently, the major events of GM plants developed and being cropped worldwide are insect-resistant and herbicide-tolerant crops (Kvakkestad 2009). Adverse effects of genetically modified (GM) plants on Lepidoptera have already been reported, which supports their quality and significance for an appropriate GMO monitoring (Graef et al. 2005). Pollen of insect-resistent Bt (*Bacillus thuringiensis*) maize toxic to pest Lepidoptera may be drifted by wind onto host plants of non-target lepidopteran larvae growing nearby (Pleasants et al. 2001, Lang et al. 2004).

Non-target lepidopteran larvae may be affected adversely by consuming this pollen attached to their host plants (e.g., Dolezel et al. 2005, Lang and Vojtech 2006, Lang and Otto 2010). Moreover, the combination of transgenic, herbicide-tolerant crops together with the application of broad-spectrum herbicides, such as glyphosate or glufosinate-ammonium, is likely to change the herbicide regime, which can reduce the weed community within fields and in field margins, in turn affecting larval and adult butterflies associated with such food plants (e.g., Haughton et al. 2003, Roy et al. 2003). Direct toxic effects of the complementary broad-spectrum herbicides on non-target Lepidoptera have received less attention, but have been reported for glufosinate (El-Ghar 1994, Kutlesa and Caveney 2001). Potentially, cultivation of the above transgenic events put at risk non-target butterflies and moths occurring in agro-ecosystems as well as protected species living in habitats near the GMO fields (Traxler et al. 2005, Hofmann and Schlechtriemen 2009, Lang et al. 2015).

Monitoring biodiversity over large areas can be inherently costly (e.g., Qui et al. 2008). As a consequence, approaches to maximize the cost-efficiency of biodiversity monitoring are highly desirable (Jones 2011), keeping in mind that cost-efficiency is not equivalent to being cheap, but means collecting data of sufficient quality with an acceptable and justified effort (Lovett et al. 2007). There will always be a trade-off between sampling costs and information quality, and the monitoring effort must be weighed against the gained results in order to make optimal use of limited resources (Haddad et al. 2008; Jones 2011; Zonneveld et al. 2003). The costs of a monitoring scheme are significantly affected by sampling intensity, i.e. by the numbers of sites or transects that should be sampled, how often transects are inspected per season, and how long line transects should be (Couvet et al. 2011; Rhodes and Jonzen 2011; Roy et al. 2007; Williams 2008). The resulting sample size, i.e. number of transects, is determined by the variance in the recorded field data, the effect size to be detected, and the desired probability to detect an effect of a given magnitude (e.g., Di Stefano 2003). Therefore, a prior analysis of expected power should always be among the first steps when planning a monitoring scheme, as this is helpful in providing guidance about the required design and sample number to detect given effect sizes (Clark et al. 2006, 2007; Elston et al. 2011; Loos et al. 2014; Perry et al. 2003).

2. Objectives

The general objective of the AMIGA Work Package 7 “Post Marketing Environmental Monitoring” is to design a comprehensive information system, methods and tools to help implement a cost-effective post-marketing environmental monitoring (PMEM) in line with the updated EFSA ERA Guidance Document (EFSA 2011).

The specific objectives of Task 7.2 “Adaptation of methodologies for Lepidoptera GMO monitoring and validation on AMIGA field sites” are:

- Development, testing and validation of a standardised monitoring approach for a GMO monitoring of Lepidoptera
- Identification of a baseline of the occurring number of lepidopteran species and their abundance in agricultural land across Europe
- Information about variance of both species richness and abundance of day-active Lepidoptera in arable land
- Estimation of the involved costs for a farmland butterfly monitoring scheme

3. Methodology

3.1 Approach and methods

Various methods exist to count and monitor butterflies, for example standardised line-transect counts, point counts, distance-sampling, or mark-release-recapture methods (e.g., Hermann 1992, Pollard and Yates 1993, Mühlhofer 1999, Sutherland 2006, Nowicki et al. 2008, VanSwaay et al. 2012). Lang et al. (2013) have published a concise summary of the methodologies with regard to the environmental monitoring of the effects of transgenic plants (including the monitoring of night-active Lepidoptera and lepidopteran larvae). The most commonly applied methodology, however, is the transect count method, often called standard "Pollard walks" (Pollard and Yates 1993, VanSwaay et al. 2012). Here, a fixed route is placed in the landscape, walked under standardised conditions, and all observed adult specimens are recorded within a defined observation area. The transect count methods has been shown to be highly adaptable, cheap and quite efficient in terms of recorded quantity and quality of data, and is easily employable by non-professional volunteers. Transect counts of butterflies is also the most applied monitoring approach in the volunteer butterfly monitoring schemes across Europe (Van Swaay et al. 2008).

Therefore, the common transect count method ("Pollard walks") is considered the best approach with regard to invested effort and recorded data (Lang et al. 2013). When implementing a standardised monitoring programme of "Pollard walks" for butterflies specifically tailored for farmland, several decisions have to be made beforehand such as the species to be recorded, the transect length, the time of year for visiting the transect, the number of visits, the time of the day, the required weather conditions, and the walking speed.). In the following the developed monitoring protocol is described in detail. This protocol is suggested for a future GMO butterfly monitoring to be installed, and was also applied in the AMIGA field tests (chapter 4, "The AMIGA field test").

Species. All adult species of the common day-active Lepidoptera are to be recorded and identified to the species level, i.e. the Papilionoidea & Hesperioidea and the Zygaenidae (Burnet Moths) (Fig. 1). Diurnal Burnet Moths are often recorded within the framework of current butterfly monitoring schemes, are threatened in some regions and provide valuable and additional information. If identification is difficult or questionable, a photograph should be taken or the specimen itself collected, depending which of the both is appropriate. Certain species groups are quite difficult to separate by species without genitalisation. In this case, the use of species pools is appropriate, also depending on the specific bio-geographical situation of the occurring species, e.g. species *Colias hyale/alfacariensis*, *Aricia agestis/artaxerxes*, *Plebejus idas/argus*, and others. In the AMIGA field test (chapter 4), all individuals of adult Snout Moths (Pyraloidea, Crambidae: Crambinae) were also recorded as a pilot test (Lang et al. 2011).

Transects. The transects should be implemented within agricultural landscapes of the respective regions (see Fig. 2), and run alongside field edges. Preferably, the transect routes follow along roads and paths, which are accessible and can be walked conveniently. The transect length is a 1 kilometre long, continuous and fixed route. The transect will be walked away and back, resulting in an overall route of 2 kilometres. Walking 1km-long transects away and back has been shown to represent a highly cost-efficient monitoring approach (Lang et al. 2016). Walking the same transect back increases sampling efficiency, and is convenient in returning to the origin (where the car may be parked). The transect itself is divided into 100 m sections, and the butterflies are recorded for each section separately (see also Appendix 1 for the field protocol). The same transects are repeated yearly.

Walks. The whole transect is walked bidirectional, i.e. from the start to the end and back again, which results in a 2 km long walk (2 times the transect length of 1 km). The transect should be walked in a slow, steady pace, and the walking speed should not exceed 3 km/h (which equals 50 m in one minute). If the walk has to be interrupted, e.g. due to bad weather conditions or when identifying a captured butterfly, counting has to be ceased while stationary.

Counts. All observed adult butterflies are identified by species and counted individually within a specified observation range. This observation range is an imaginary box, 2.5 m to each side and 5 m in front and above the recording person (Fig. 3). The numbers of butterflies are written down per species (by their scientific name) using the provided field protocol (Appendix 1). Separate protocol sheets are to be used for the recordings on away and back path, so that the specimens can be attributed accordingly. The butterflies must be recorded per 100 m sections of the transect (see field protocol, Appendix 1)

Fig. 1. Example for species to be recorded in a farmland monitoring programme (from left to right): Common Swallowtail (*Papilio machaon*, Papilionoidea), Lulworth Skipper (*Thymelicus acteon*, Hesperioidea) and Six-spot Burnet (*Zygaena filipendulae*, Zygaenidae) © A. Lang.

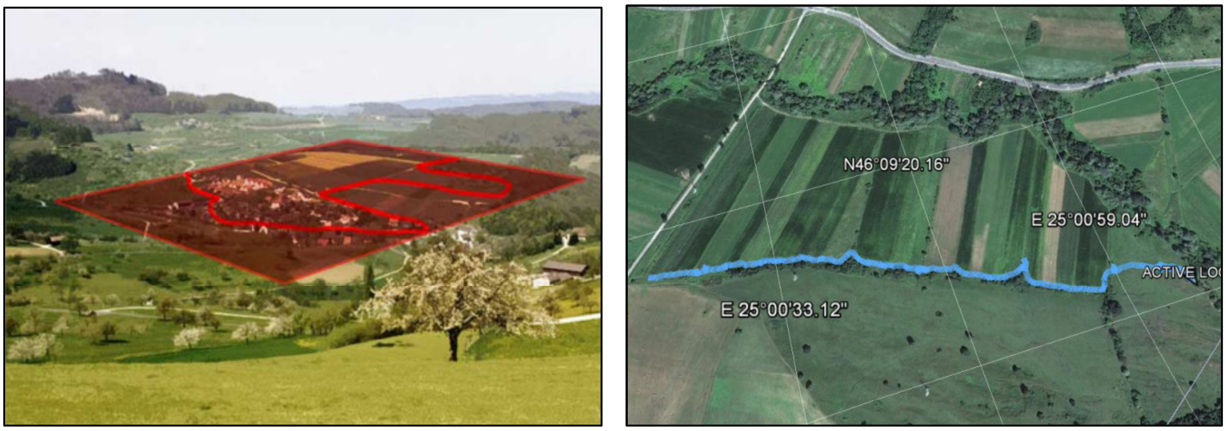


Fig. 2. Example for a butterfly transect route of the Swiss national biodiversity monitoring (left, © Hintermann&Weber AG), and an AMIGA butterfly transect in Romania (right). A fixed route, the transect, is placed in the landscape along arable fields. The transect is walked with defined speed and all butterflies observed in a specified observation range are recorded.

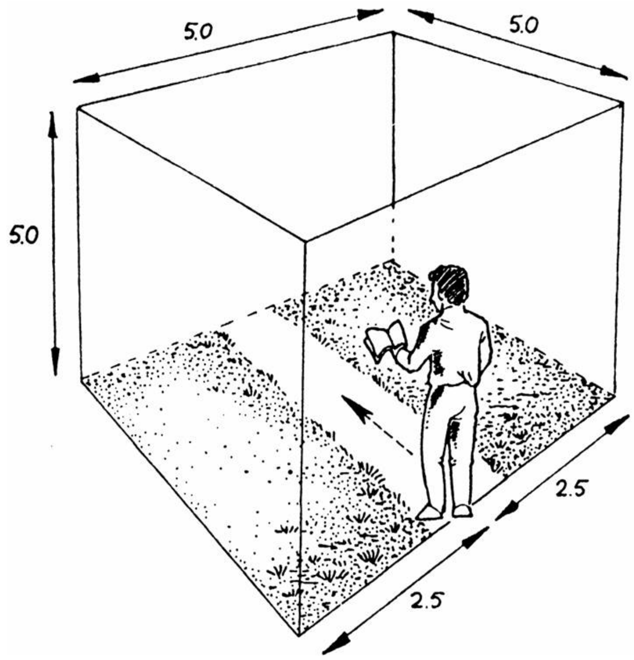


Fig. 3. The "observation box", an imaginary space of 2.5 m to each side and 5 m in front and above, in which every observed individual butterfly will be recorded (after TMD 2007).

Conditions. For the recordings, good weather conditions must be met with regard to wind, temperature and cloud cover. The weather conditions must be recorded (see Appendix 1 for field protocol). In general, counts are only allowed in "nice and pleasant weather" (Van Swaay et al. 2012). Counts are only allowed when the air temperature is above 13°C (measured in 1 m height in the shade). Between 13°C and 17°C the cloud cover should be less than 50%, while above 18°C it is also possible to monitor with higher cloud cover. It is recommended to stop recording when larger bank of clouds pass by, and to resume counting after they have passed. The wind speed should not be above 3 Bf on the Beaufort scale (i.e., 3.4 – 5.5 m/s). Short, stronger gusts can be tolerated, e.g. on exposed hills, but if the wind speed exceeds 3 Bf for longer periods, the recording must be interrupted or terminated.

Number and time of visits. Each transect is visited at least four times per season. Two summer visits (e.g. June, July) should always be included, one visit in spring (e.g. April/May) and one visit towards the end of summer (July/August) should complete the recording. Spreading the visits over the season and including summer months guarantees a sufficient capture efficiency of the species (Lang et al. 2016). However, it can be advisable to add a fifth visit depending on the local situation. Reduced effort schemes of 4 – 5 visits per season have been shown to still record the majority of butterflies as compared to a higher effort scheme (Roy et al. 2007, Jonason et al., 2010, Hardersen and Corezzola 2014). For a farmland monitoring of butterflies and burnet moths Lang et al. (2016) also recommended 4 (– 5) inspections per transect per season. Transect counts should take place during the hours that the butterflies are mostly active, e.g. between 10 am and 5 pm in Central Europe. The date of visits during the season and the time of day when visits take place will strongly depend on the geographical location and the altitude of the respective site, and, if necessary, can be adjusted to these local conditions.

Additional recordings. More general background information about each transect is to be provided in a cover sheet (see example in Appendix 2). Additional recordings include the flowering aspect on the transect, the habitat types bordering the transect, any other observation relevant for the occurrence of butterflies, and the GPS coordinates of the transect (see Appendices 1 and 2, and the following section).

Data collection. All collected and observed data are transferred from the field protocols to e.g. Excel sheets, and delivered to the co-ordinator at the end of each season after the last transect visit.

3.2 Sampling protocol

In order to standardise the data collecting, default data and parameters have to be recorded by means of a standard protocol. Following a normed protocol scheme will standardise and facilitate the data collection and analysis, thus assuring data quality (cf. VDI 2010, Lang et al. 2013). Printed sheets can be used for data collection (see Appendices 1 and 2), and the data digitalised later, e.g. in EXCEL or any other database. However, it is recommended to use an app for mobile devices for the data collection in the field (see below).

Sampling protocol
In the header of the field monitoring protocol, corresponding data will be given such as location, date and time, weather conditions, recorder, and the like (Appendix 1). All observed individuals will be identified to species level and noted separately for each transect section.

The flowering aspect of the transect must be noted per 100 m section, as flower density will significantly affect butterfly numbers (Lang et al. 2011). Density of flowering nectar plants relevant for butterflies must be allocated to one of four possible classes: missing/inferior, low/poor, average, large/high (see field protocol, Appendix 1).

Any other observation relevant for the occurrence of butterflies should be noted under comments, e.g. previous mowing of grassland or agricultural management practices.

On a second sheet (Appendix 2), further relevant information on the site and transect is recorded:
- Corresponding information with respect to the transects should be noted such as location, altitude, exposition, GPS coordinates, description of the landscape and field boundaries, etcetera (see Appendix 2 for an example).
- One additional walk per season should be done in order to record the habitat types that the transect runs through. Habitat types should be recorded per 100 m section of the transect, and follow the EUNIS classification (http://eunis.eea.europa.eu/habitats-code-browser.jsp). In general, a course classification of the habitat type is sufficient (see example in Appendix 3). If an arable field is bordering the transect, the specific crop type and its proportion must be recorded in addition.
- The transect must be indicated on a map, e.g. on a google map, marking it with a line (see also example in Fig. 2). The coordinates of the start and the end point of the transects must be recorded, preferably by GPS. Ideally, the whole transect route is tracked with a GPS (see Fig. 2).

App for mobile devices
It is highly recommended to use an Android app for mobile devices for the field recording instead of writing the observation in paper protocols. Using an app guarantees that recorders follow a prescribed structure of data input which much reduces the subsequent investment of supervisors in controlling and revising the field data. For instance, regional species lists could be built into the app. Moreover, the data are already digitalised and must not be transferred tediously from paper to databases by hand. In addition, location, time, walking, speed can be recorded automatically, weather conditions can be incorporated from the next weather station, and the GPS coordinates will be registered for each single butterfly observation as well as the GPS track of the transect route on a map. For several years now, an app for mobile devices is

successfully used within the Swiss national biodiversity monitoring programme BDM (Fig. 4). The development costs for such an Android app are reasonable, presumably in the range of 20000 Euro (offer by a software company requested in 2014). Meanwhile, several Android apps for recording butterflies are also available as free-ware, e.g. the Unified Butterfly Recorder (UBR) developed by entomologists of the Iowa State University, available at: http://www.reimangardens.com/collections/insects/unified-butterfly-recorder-app/.

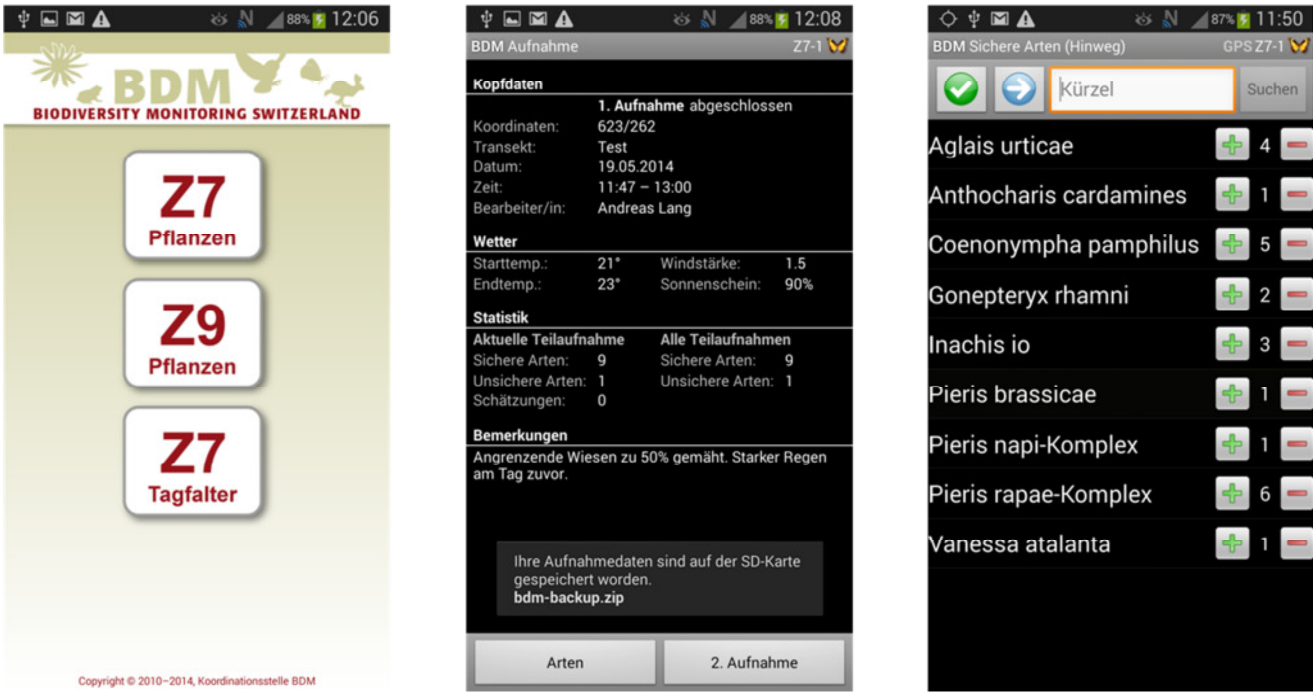


Fig. 4. Screenshots of the Android app used in the Swiss national biodiversity monitoring programme BDM (© Koordinationsstelle BDM).

4. The AMIGA field test

The developed sampling protocol described in the previous section was put to the test and validated in three European countries, i.e. a butterfly monitoring was carried out in farmland of Sweden (Scania), Spain (Catalonia) and Romania (Transylvania), for three field seasons. The first two years (2013, 2014) were funded by AMIGA, and the third season (2015) by INRA, France, and the FAG of the University of Basel (Freiwillige Akademische Gesellschaft Basel), Switzerland. Selected butterfly transects according to the above description were established in farmland with a high proportion of maize crops, resulting in 10 – 11 transects per country. The general conditions of the butterfly monitoring are summarised in Table 1, for the detailed methods refer to the previous chapter. Local and professional butterfly experts were contracted to carry out the monitoring. The involved organisers and field recorders of the AMIGA butterfly monitoring are shown in Table 2.

Table 1. Summary of the basic framework of the AMIGA butterfly monitoring scheme.

Aspect	Details	Comment
Countries	Spain, Romania, Sweden.	Representative for biogeographic regions across Europe: "Mediterranean" (Spain), "Continental" (Romania) and "Atlantic/Continental" (Sweden).
Sites	In agriculturally managed land.	Including a high proportion of maize crop.
Duration	2013 – 2015	Funded by AMIGA (2013, 2014), and INRA and University of Basel (2015).
Number of transects	At least 10 transects per country.	Eleven transects in Spain.
Number of counts	4 counts per season and transect.	Actual dates and time of inspections depending on the region.
Transect length	1 km long	Transect divided in 100 m sections; transect route walked away and back.
Recorded species groups	Papilionoidea and Hesperioidea, Zygaenidae (and Crambinae)	By use of a standard field protocol.
Additional recording	Estimation of flower density along transects.	Recorded per 100 m section.
Additional recording	Habitat characterisation.	According to EUNIS classification of habitat types, per 100 m section.
Additional recording	Recording of average working hours required per transect.	In 2014 (and partly in 2015).

Table 2. Involved collaborators organising and conducting the AMIGA butterfly monitoring 2013 – 2015.

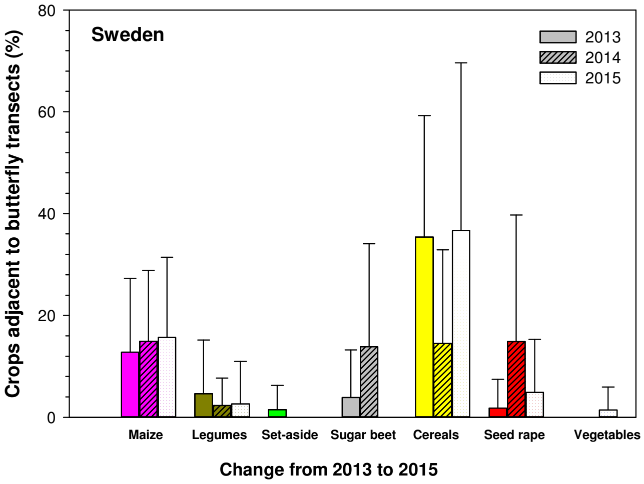
Name	Affiliation	Monitoring in	No. of transects
Constanti Stefanescu	Granollers Museum of Natural Sciences, E-08401 Granollers	Spain	8
Marina Lee	University of Lleida, E-25003 Lleida	Spain	3
Mikael Molander	Hexapoda Konsult, SE-27539 Sjöbo;	Sweden	10
Lars Pettersson	Lund University, SE-22362 Lund	Sweden	
Laszlo Rakosy	Babes-Bolyai University, RO-3400 Cluj	Romania	4
Iulia Muntean		Romania	
Jacqueline Loos	Leuphana University, D-21335 Lüneburg	Romania	3 (2013/14)
Andreas Lang	Büro Lang, D-79669 Zell i.W.	Romania	3
Franz Kallhardt		Romania	(6 in 2015)

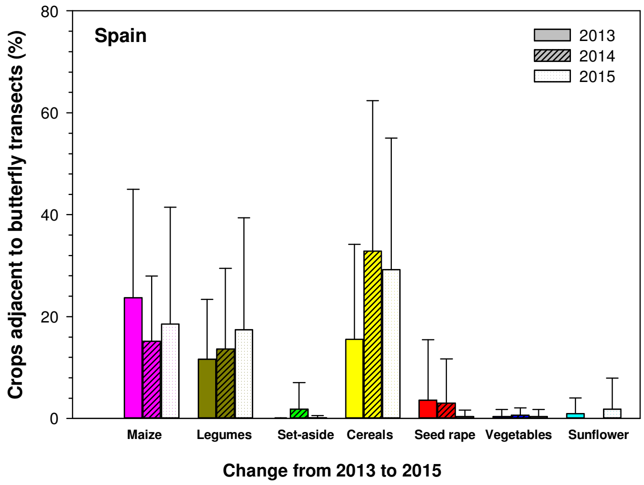
4.1. Habitats and crops

Habitat types and crops bordering the AMIGA butterfly transects were recorded according to the EUNIS classification (see Appendix 3). Recorded habitat types were summarised to classes broader than the original EUNIS definitions in order to enable a comparison between countries (Table 3). In all countries, roughly 2/3 of the neighbouring habitats were arable fields; taking into account grassland the adjacent agricultural land amounted to 75% – 90% (Table 3). Neighbouring habitats were quite stable and changed only marginal from year to year (Table 3).

Table 3. Proportion (%) of habitat types bordering the AMIGA butterfly transects in Sweden, Spain and Romania (mean ± SD).

Country	Grassland	Crop fields	Orchards	Forest fringes	Trees, bushes	Reeds	Gardens	Buildings	(Rail)roads
Sweden									
2013	29.42 ± 19.30	60.09 ± 18.05	0	4.45 ± 9.38	1.80 ± 3.07	0	1.47 ± 2.56	0	2.77 ± 6.00
2014	28.67 ± 20.76	60.83 ± 19.90	0	4.45 ± 9.38	1.80 ± 3.07	0	1.47 ± 2.56	0	2.77 ± 6.01
2015	29.00 ± 19.85	60.50 ± 20.19	0	4.45 ± 9.38	1.80 ± 3.07	0	1.47 ± 2.56	0	2.77 ± 6.01
Spain									
2013	7.56 ± 12.98	67.37 ± 26.09	9.15 ± 23.75	16.30 ± 21.31	4.23 ± 8.65	0.45 ± 1.50	0	0.63 ± 1.56	0
2014	9.10 ± 13.26	67.69 ± 28.58	9.15 ± 23.75	10.40 ± 11.85	2.73 ± 7.64	0	0	0.90 ± 2.02	0
2015	9.10 ± 13.26	67.28 ± 28.35	9.15 ± 23.75	10.40 ± 11.85	2.73 ± 7.64	0	0	1.32 ± 3.10	0
Romania									
2013	18.73 ± 20.17	56.40 ± 18.07	4.25 ± 13.44	7.50 ± 13.44	8.05 ± 9.78	0.55 ± 1,21	0	0	4.45 ± 14.07
2014	19.45 ± 20.74	56.00 ± 18.02	4.50 ± 14.23	7.35 ± 13.45	6.05 ± 8.30	0.45 ± 1.12	0	0	4.50 ± 14.23
2015	19.00 ± 15.70	56.30 ± 12.55	4.50 ± 14.23	7.65 ± 13.42	7.45 ± 8.21	0.67 ± 1.35	0	0	4.50 ± 14.23





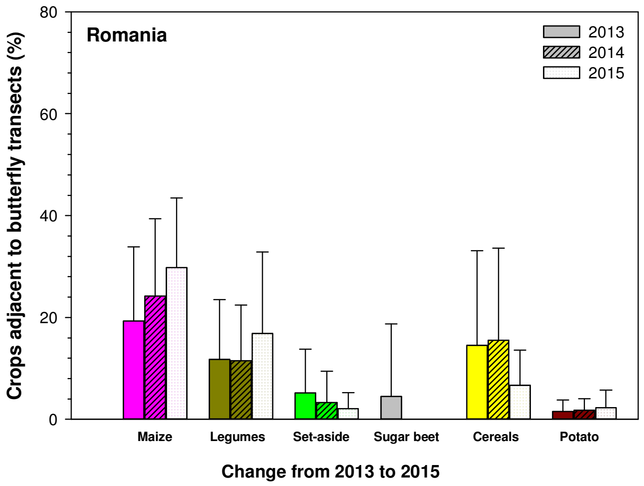


Fig. 5. Crop types adjacent to the AMIGA butterfly transects in Sweden, Spain and Romania from 2013 – 2015 (means + sd).

Maize was a major crop in the study regions. In Sweden and Spain, cereals were the dominant crop and maize the second most frequent one (Fig. 5). In Romania, maize was the prevailing crop followed by cereals and legumes. Corresponding to crop rotation, the proportion of the different crops could change substantially from year to year, and at least in one year maize was the dominant crop in all countries. In Romania there seemed to be a trend for expanding maize cultivation in the three observed years (Fig. 5). Circumstantial observations indicated an increasing intensification of agricultural management in Romania, exemplified by clearances of hedgerows and ploughing of grassy field margins.

4.2 Butterfly and Burnet Moth species

The recorded species richness and abundance of Lepidoptera (butterflies and burnet moths) in farmland was highest in Romania. Overall, 102 species and 16’856 individuals were recorded on the 10 transects installed in the Romanian agro-ecosystems (Fig. 6). Overall, 82 species and 7’852 individuals were recorded on the 11 transects in Spain, and in Sweden 30 species and 7’501 individuals on 10 transects (Fig. 6). Consequently, average species number and abundance per transect was highest for Romania, followed by Spain and Sweden (Fig. 7). In Appendices 4 – 6 the recorded species are listed for the different countries. The species number was fairly stable from year to year in all countries, but individual abundance showed considerable annual fluctuations (Figs. 6, 7). Although the butterfly community differed between the countries, 18 common species occurred in all three countries (see Appendices 4 – 6).

In Romania, 10.6% of the recorded butterfly species (excl. Zygaenidae) are listed in the European Red List (Van Swaay et al. 2010), 8.6% of the species in Spain and none in Sweden. In Romania, 34% of the recorded butterfly species (excl. Zygaenidae) are listed in the national Red List (Rakosy 2002). In Sweden, 6.5% of the recorded species (incl. Zygaenidae) are listed in the Swedish Red List (Ahrné 2015). In Spain, the national red list (Lista roja de los invertebrados) is too limited for butterflies and not applicable for the time being.

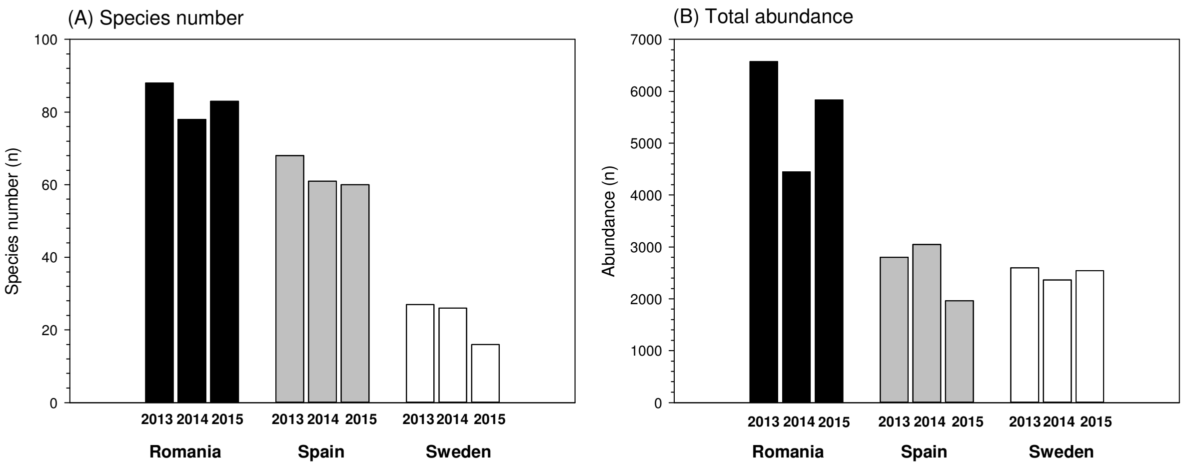


Fig. 6. Species number (A) and total abundance (B) of butterflies and burnet moths (Lepidoptera: Papilionoidea, Hesperioidea, Zygaenidae) of the AMIGA transects in Romania, Spain and Sweden (2013 – 2015).

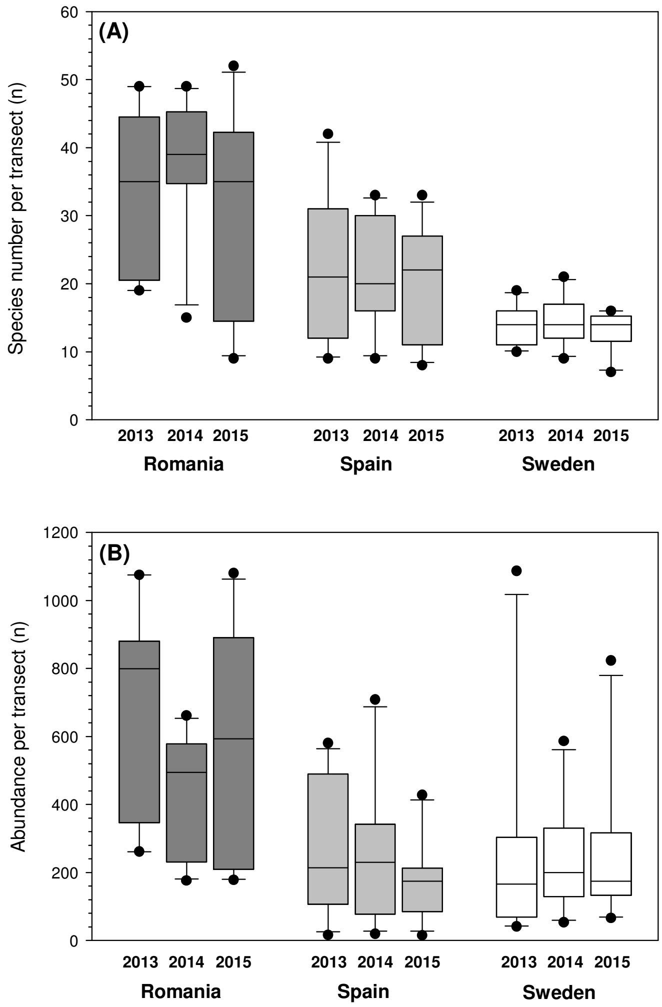


Fig. 7. Species number (A) and total abundance (B) of butterflies and burnet moths per transect, country and year of the AMIGA monitoring. Boxes show the 25% and 75% quartiles, the horizontal line within the box is the median, while 10% and 90% percentiles are indicated by the whiskers, and outliers by dots.

Recording Snout Moths (Pyraloidea, Crambidae: Crambinae) had been suggested as an additional indicator group for a GMO monitoring (Lang et al. 2011). During the AMIGA field monitoring a total of 611 individuals was recorded in Romania, 3 individuals in Spain, and 709 in Sweden. In Spain, the Snout Moths seem not to provide additional information value due to their too low numbers. But even in Romania and Sweden the interpretation of Snout Moth numbers was problematic, because their abundance could be quite variable from year to year (only 6 % of the total catch in Romania in year 2015), and differences between transects were large (0 – 169 individuals per transect in Romania, 0 – 74 individuals per transect in Sweden). The AMIGA approach to walk the transects on roads and paths possibly impeded a standardised and more effective recording of Snout Moths, which preferably thrive on grassy surface and only fly up on approaching closely. The development of a standardised recording protocol specifically for Snout Moths appears necessary taking into account the behaviour of Snout Moths.

The recorded species numbers of butterflies and burnet moths increased with the length of the transects showing a similar general pattern, though different figures, between years and countries (Fig. 8A). It appears that in Sweden a plateau is reached at a length of 2 km, which is likely due to the lower amount of species present in Swedish arable land (in other words, there are not many new species to be detected). In contrast, the transect-species curves in Romania and in Spain seem still be increasing above 2 km, especially in Spain, although a slight levelling off can be observed above 1 km. As transects were walked in both directions, the recorders turned at 1 km and walked the same route back (see "3.1 Approach and methods"). Walking transects in both ways improved sampling efficiency: on the route back (1 km – 2 km), on average 13% – 23% of the species were observed, i.e. species that had not been recorded on the away walk (Fig. 8B). Further, walking transects bi-directionally (away/back) allows to calculate the detectability of species, which, in turn, provides the opportunity to estimate population trends more rigorously (e.g., Kéry and Plattner 2007).

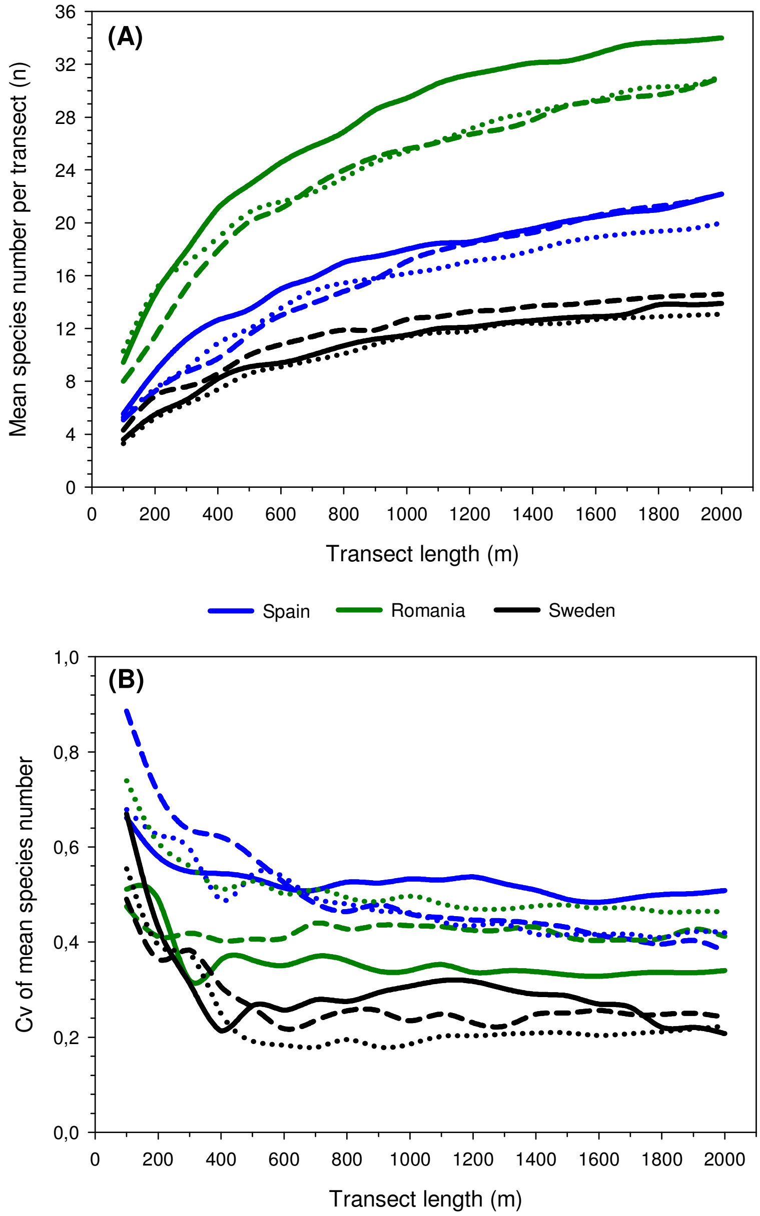


Fig. 8. (A) Relationship between transect length and observed number of species per transect (average of 10 – 11 transects). (B) Relationship between transect length and coefficient of variation of species number. Different countries are shown in different colours, and different years in different patterns (2013: straight lines; 2014: dashed lines; 2015: dotted lines).

The degree of variance of the data set is relevant for statistically detecting a (GMO) effect on lepidopteran species number, i.e. the higher the variance of the data the lower is the likelihood to detect an effect (or in other words, a higher sample size, i.e. number of transects, is necessary to detect a given effect). There were clear differences between countries: Spain showed the highest CV (coefficient of variation) in species number, Sweden the lowest, and the variance in Romania was intermediate (Fig. 8B). The pattern of the relationship between the CV of species number and transect length was relatively consistent within countries between different years, but the actual degree of the CV can be quite different between single years (Fig. 8B). In general, the CV of species number dropped above 400 – 800 meter transect length, and remained fairly constant thereafter (Fig. 8B), suggesting that for statistical reasons transects should be at least longer than 800 m when studying Lepidoptera species number in farmlands.

5. Power calculations

Approach

The number of transects (sample size) is a crucial factor determining the monitoring costs of a butterfly monitoring, once the transect length and the number of inspections per season are fixed. The number of required transects is affected by the desired statistical power, the significance level α, the effect size to be detected and the variance of the data set, i.e. the standard deviation of the population (Table 4).

Table 4. Parameters determining the statistical power (from Nakagawa & Foster 2004).

Table 1 The type of change required in the size of the significance criterion, the sample, and the effect size to increase statistical power

Statistical parameters	To increase statistical power:
Significance criterion (α)	Increase α
Sample size (N)	Increase N
Effect size (d)	Increase $\lvert d \rvert$[a]
Effect size d is composed of two parts ($d=[\mu_t-\mu_c]/\sigma$)	
Difference between population means ($[\mu_t-\mu_c]$)	Increase $\lvert \mu_t-\mu_c \rvert$[a]
Population standard deviation (σ)	Decrease σ

[a]These are absolute values

Here, we conducted prospective power analyses in order to estimate the number of transects needed to detect changes of a given magnitude (= effect size) on species number or abundance of Lepidoptera. Regarding abundance, we tested the total abundance as well as abundance of selected species pools such as Red List species, grassland species and common species. "Common species" comprised the 18 species which occurred in all three study countries (cf. Appendices 4 – 6), "Red list species" were either selected from the European Red List (Van Swaay et al. 2010) or from the national red list of Romania (Rakosy 2002), and "Grassland species" were the indicator species of the European Environment Agency (EEA) used for the monitoring of European grassland butterfly species (Van Swaay et al. 2015) (see also Table 5).

The freeware programme G*Power, version 3.1, was used for the calculations of the statistical power analysis (Faul et al. 2007). The required sample size was calculated for changes ranging from 10 % to 50 % of Lepidoptera species richness or abundance. We based all power analyses on a two-sided test with a significance level of p = 0.05 and a power of 80 %, which is suggested to be adequate (Di Stefano 2003, Perry et al. 2003, Lang, 2004). We assumed a paired t-test analysing lepidopteran numbers on the same transects, because dependent samples were shown to be more powerful and cost-efficient (Lang & Bühler 2012). The standard deviations for the lepidopteran populations were taken from the AMIGA field results (see "4.2 Butterfly and Burnet Moth species"). In a dependent comparison, the correlation of the data from year 1 (census 1) to year 2 (census 2) affects the power of the test, i.e. the stronger the correlation the more powerful the statistical test. For the power calculations, the degree of the data correlation was also derived from the AMIGA field results and is shown in Table 5.

Table 5. Correlation coefficients (Spearman's rank, $p < 0.01$) for the association of Lepidoptera species number and abundance of different lepidopteran groups between sampling years. EU-RL species = species listed in the European red list (Van Swaay et al. 2010); Nat-RL species = species listed in the red lists of Romania (Rakosy 2002) and Sweden (Ahrné 2015); Grassland species = species of the European Grassland Butterfly Indicator (Van Swaay et al. 2015); Common species = 18 common species recorded in all three countries Romania, Spain and Sweden during the AMIGA study (see Appendices 4 – 6); na = not applicable due to low numbers.

Country year 1-year 2	**Species number**	**Total abundance**	**EU-RL species**	**Nat-RL species**	**Grassland species**	**Common species**
ROMANIA						
2013-2014	0.91	0.76	0.61	0.72	0.78	0.82
2014-2015	0.96	0.89	0.36	0.69	0.81	0.85
SPAIN						
2013-2014	0.95	0.83	0.90	na	0.86	0.66
2014-2015	0.96	0.95	0.97	na	0.96	0.74
SWEDEN						
2013-2014	0.62	0.95	na	na	0.89	0.93
2014-2015	0.34	0.91	na	na	0.96	0.89

Results

Preliminary considerations

In an initial analysis it was assessed, how sample size, i.e. number of studied transects, affects the accuracy of estimating the coefficient of variation (CV). For this purpose, the dataset of Lang & Bühler (2012) was used, which consisted of 86 transects (each 2.5 km long), situated in farmland in Switzerland. The calculated coefficient of variation was 0.29 for species number of butterflies and burnet moths (Papilionoidea, Hesperioidea, Zygaenidae). Then, it was tested how precise the known CV could be assessed with differing numbers of transects: we randomly selected 3, 5, 7, 10, 15, 20, 25, 30, 35 or 40 transects from the overall dataset, and calculated the CV from these subsamples. Each subsample was repeated 10 times.

Not surprisingly, the more transects were counted the more precise was the assessment of the CV (Fig. 9). Ten transects appeared to represent a threshold level above which the assessment was relatively close to the correct CV. Therefore, it was decided to implement 10 transects in each country (Romania, Spain, Sweden) giving a total sample size of 30 transects for the AMIGA butterfly monitoring study.

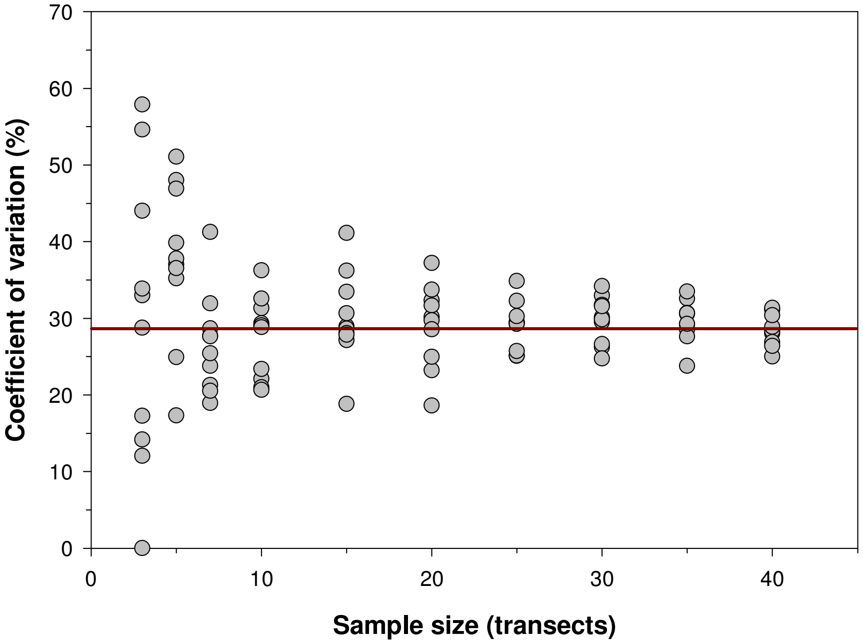


Fig. 9. The effect of sample size (number of transects) on estimating the accurate coefficient of variation of 29% (red line) for species number of butterflies and burnet moths in farmland (Switzerland, dataset of Lang & Bühler 2012).

Power analyses

The required number of transects to detect a given effect differed among various variables or endpoints, respectively (see Figs. 10 – 11). For instance, about 13 – 27 transect have to be sampled to detect a 10% loss of species number (Fig. 10A). Similarly, less than 20 transects have to be sampled to detect a 30% reduction of total abundance (Fig. 10B). The differences between the three countries tested were remarkably small. However, in any year and country outliers could increase sample size by a factor of 2 – 10, e.g. up to 56 transects for a 10% species loss in Sweden 2014 (Fig. 10A), or up to 63 transects to detect a 30% reduction in total abundance in Sweden 2013 (Fig. 10B).

Sampling only specific, selected subgroups of species and analysing effects on their abundance would increase necessary sample size (Fig. 11). For detecting a 30 % reduction in abundance, the required number of transects increased roughly by 15 % for common species, by 60% for the EEA grassland butterfly indicator species, and by 30 % for red list species (Fig. 11) as compared to sampling for total species number or total abundance (Fig. 10). In other words, the statistical power for analysing effects on species pools will be lower given a fixed number of transects. Again, occurring outliers could enhance necessary sample size considerably (Fig. 11).

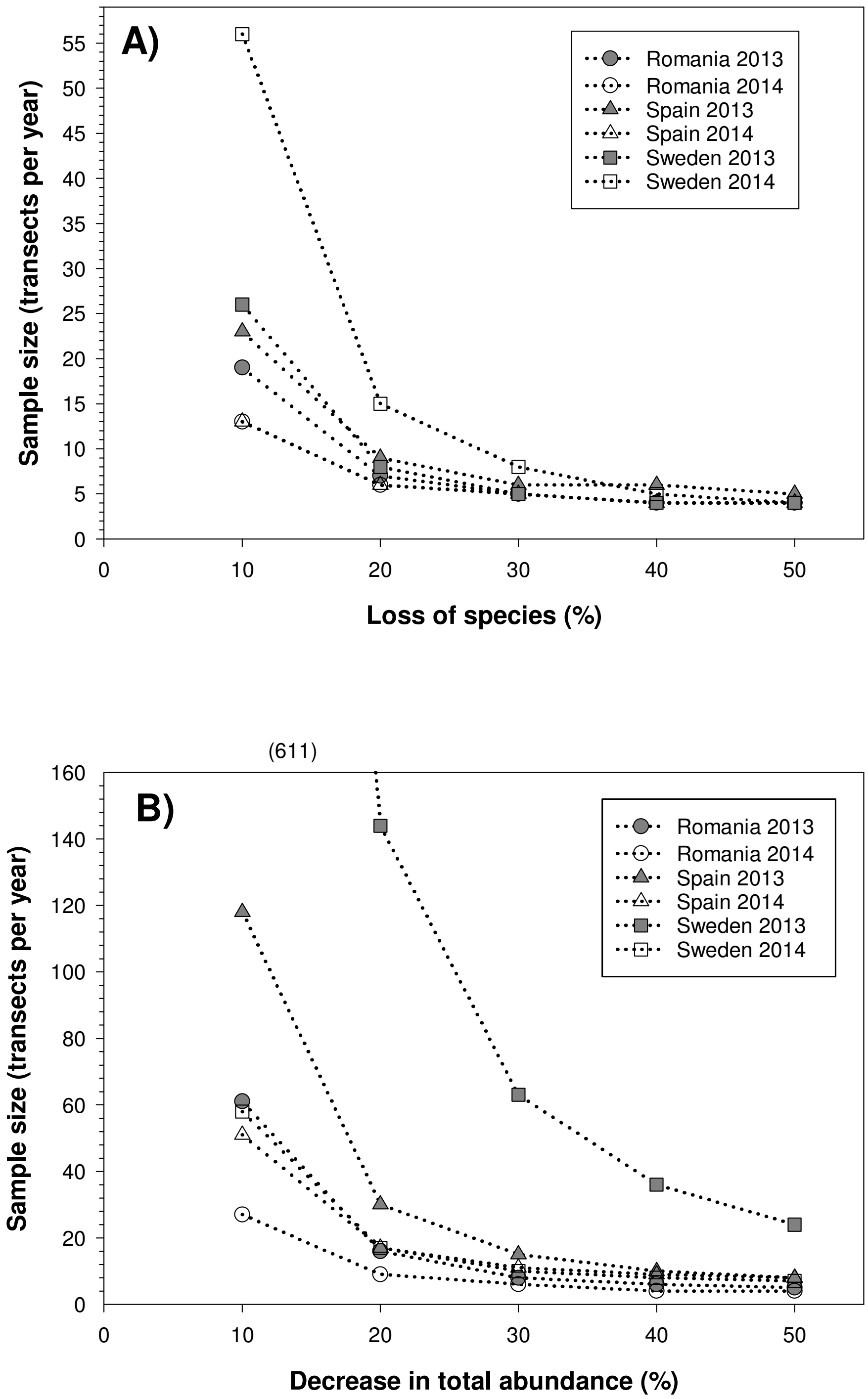


Fig. 10. Number of required transects for detecting an effect on mean species number (A) or on total abundance (B) of diurnal Lepidoptera. Sample size estimation (number of transects) is related to different scenarios ranging from a 10 % reduction to a 50 % reduction in species number or abundance. The y-values out of range are indicated in brackets above the figure. For further explanations and underlying assumptions of the power analyses see text above (in section “Approach”).

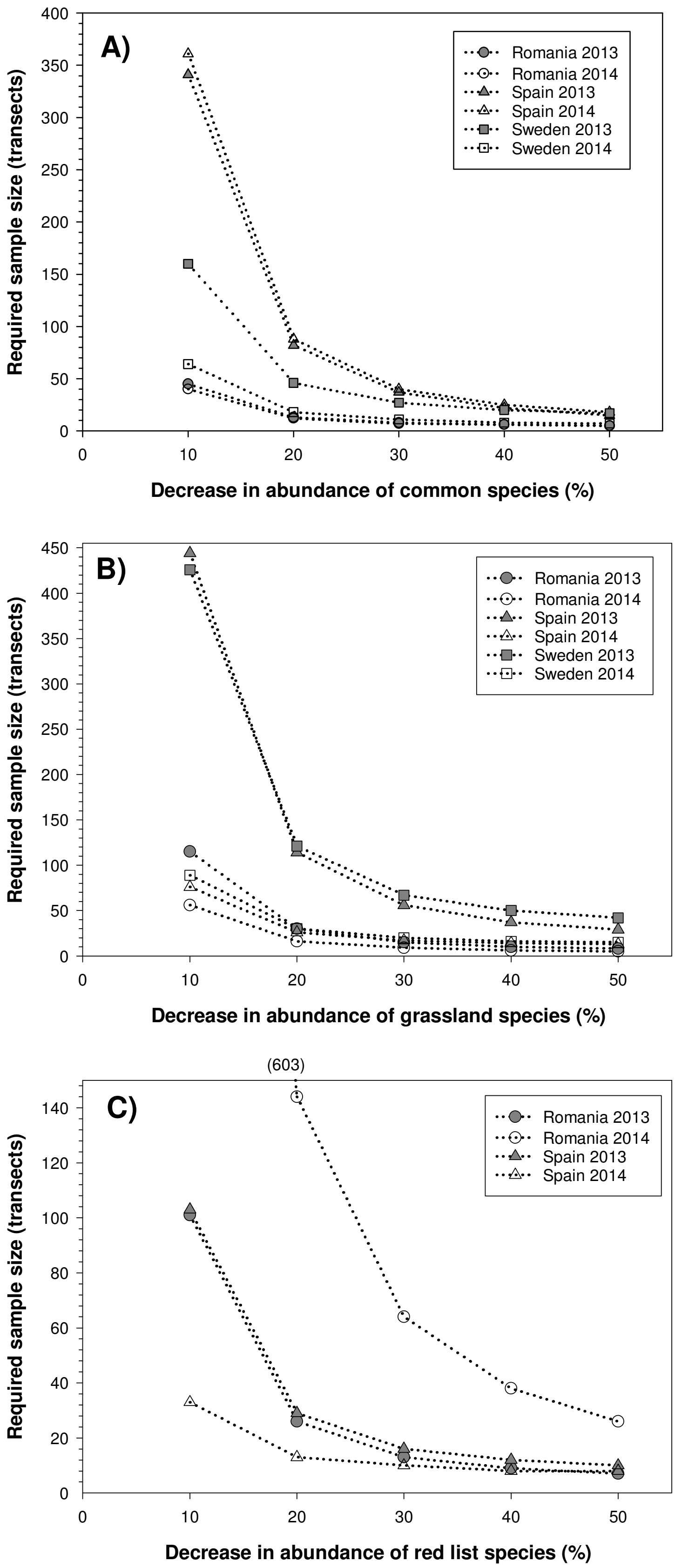


Fig. 11. Number of required transects for detecting an effect on total abundance of “common species” (A), “grassland species” (B) and “red list species” of diurnal Lepidoptera. Sample size estimation (number of transects) is related to different scenarios ranging from a 10 % reduction to a 50 % reduction in abundance. The y-values out of range are indicated in brackets above the figure. For further explanations and underlying assumptions of the power analyses see text above (in section “Approach”).

6. Monitoring effort

Approach

The time effort required on transects was documented for various activities. When working the transects, the involved time for the following variables were recorded for each transect: driving time to the transect from home, driving time between transects, walking time on the transects in order to count butterflies, time for additional other work (i.e., finding the transect, identifying butterflies at home, preparations and organisation, field activities other than recording butterflies and describing habitat), transfer of field protocols into EXCEL sheets, and time for the habitat description.

When calculating the overall time effort for a butterfly monitoring scheme, we assumed that per day two transects can be handled (supposing an 8-hour working day), and that four inspections will be done per year. We compared the resulting effort between countries and years by multiplying the recorded working time by the number of transects indicated by the above power analyses (see "5. Power calculations"). Although the driving time to transects differed between countries, we used the same mean value for all countries in order to standardise the travel time. For all other variables we used the actual recorded values, e.g. for walking transects or the habitat description, because these variables were truly country-specific due to the differing butterfly communities and environmental conditions.

The calculated time effort refers to the monitoring effort of one year. It has to be noted that a dependent sample design was assumed for the power analyses, i.e., the transects must be sampled at least twice. Thus, the calculated time effort must be multiplied by the number of censuses or years, respectively, that the transects will be sampled. The following activities were not included in the calculated time effort: general organisation of the monitoring, supervisor activities such as instruction and control of field workers, data processing and control by supervisor, statistical analysis of data or written reports.

Results

The time to be invested into a butterfly monitoring scheme of the specified design effort differed between the countries. Butterfly numbers were much higher and the habitats more diverse and dynamic in Spain and Romania, consequently field work and data import took more time than in Sweden (Table 6). For calculating the overall effort, the mean value was taken for driving and travelling time, and the actual values for all other variables, resulting in a total sum of 19.13 hours for Sweden, of 27.67 hours for Spain, and 29.34 hours for Romania (for 2 transects per working day and with 4 inspections per year).

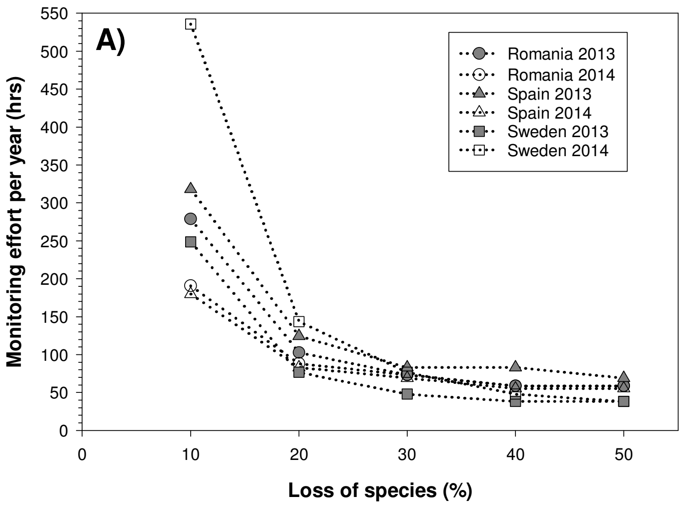
Table 6. Observed effort (minutes) for a monitoring design for different countries (for two transects walked per working day and 4 inspections per year). The values are arithmetic means of 10 (Sweden, Romania) and 11 transects (Spain).

Country	Driving time [1)]	Travel between transects [2)]	Walking transects [3)]	Other work [4)]	Data import [5)]	Habitat description [6)]	Total
Sweden	332.00	168.00	387.60	101.84	216.00	40.00	1245.44
Spain	352.73	68.00	620.56	258.18	320.00	58.76	1678.23
Romania	220.40	66.67	766.60	180.00	332.00	79.00	1644.67
Mean	301.71	100.89	591.59	180.01	289.33	59.25	1522.78

[1)] Driving time to the transect(s) and back home. [2)] Driving time from the 1st to the 2nd transect. [3)] According to field protocols. [4)] Finding the transect, identifying butterflies at home, preparations & organisation, field activities other than recording butterflies and describing habitats. [5)] Transfer of field protocols into EXCEL sheets. [6)] Habitat description was required only once per transect and year.

The time effort to be invested in a butterfly monitoring follows the required sample size, for a given region this generally varies between 180 and 318 hours per year, or 22 to 40 working days, in order to detect a 10 % loss in species number (Fig. 12A). To detect a 30% reduction in total abundance would require 88 – 207 hours per year, or 11 to 26 working days, in general (Fig. 12B). But in any year and region outliers can increase the necessary time investment. For example, in Sweden in the year 2014 detecting a species loss would have required considerably more time (Fig. 12A), and analysing the total abundance in Sweden and Spain both in 2013 would have also involved much more effort (Fig. 12B).

At first instance it may surprise that necessary time effort is not lower in Sweden as compared to the other two countries (Fig. 12), considering that the CV is lowest for species number (Fig. 8), and the actual monitoring effort on the transects is much lower (Table 6). However, year-to-year variation was large for species number and butterfly abundance in Sweden, either resulting in a low correlation coefficient for species number (Table 5), or a high CV for total abundance (data not shown). In the end, the resulting monitoring effort is relatively similar for the three countries due to the different interacting factors affecting the statistical power.



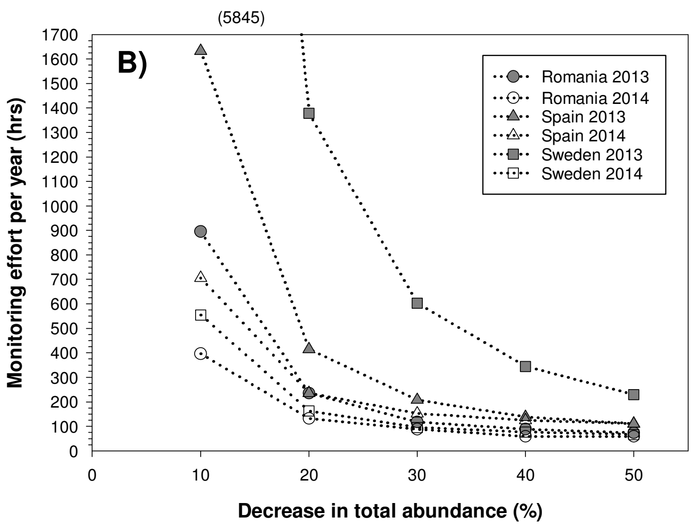


Fig. 12. Monitoring effort (hours) per year for detecting an effect on mean species number (A) or on total abundance (B) of diurnal Lepidoptera. Monitoring effort is related to different scenarios ranging from a 10 % reduction to a 50 % reduction in species number or abundance. The y-values out of range are indicated in brackets above the figure. For further explanations and underlying assumptions of the calculations see text (section "Approach").

Targeting the monitoring of specific species groups would require more effort as exemplified by the pools of common species, EEA grassland butterfly indicator species and red list species (Fig. 13).

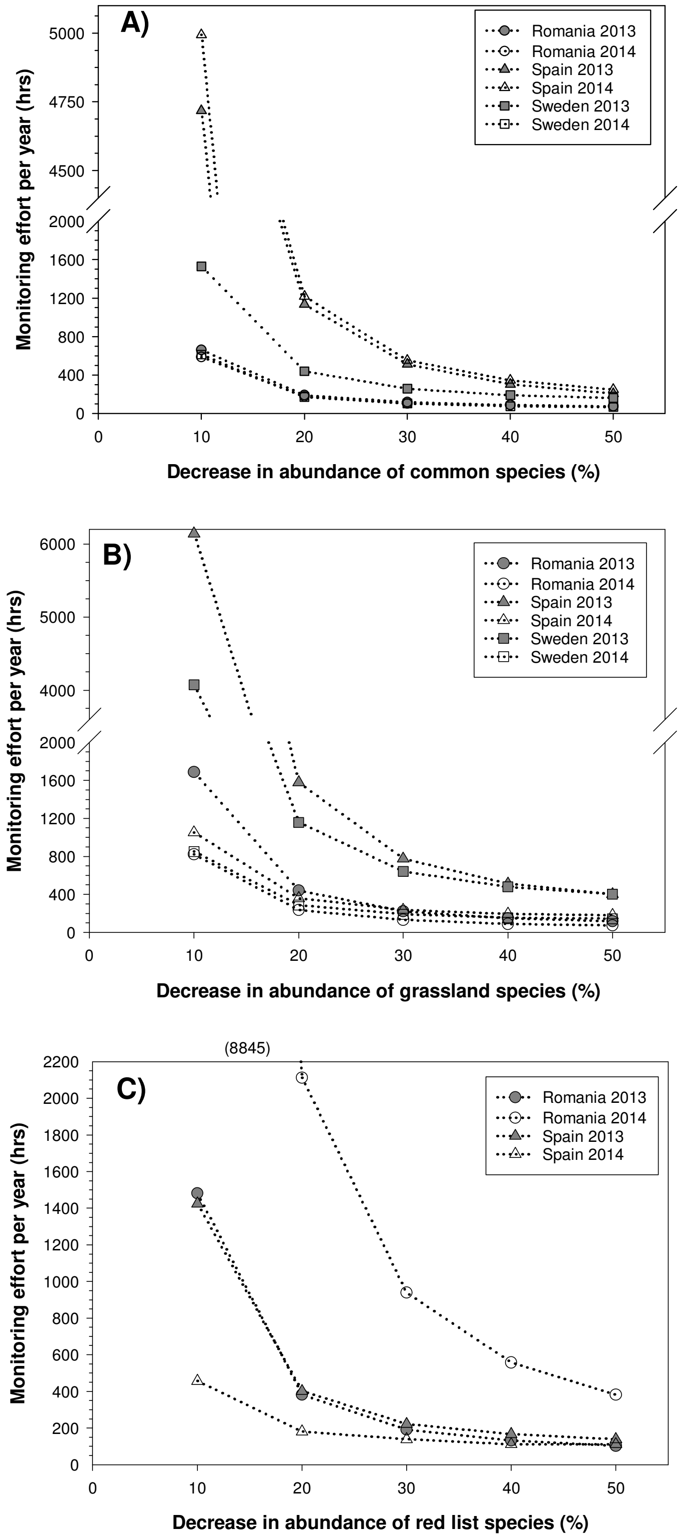


Fig. 13. Monitoring effort (hours) per year for detecting an effect on the abundance of "common species" (A), "grassland species" (B) and "red list species" (C) of diurnal Lepidoptera. Monitoring effort is related to different scenarios ranging from a 10 % reduction to a 50 % reduction in species number or abundance. The y-values out of range are indicated in brackets above the figure. For further explanations and underlying assumptions of the calculations see text (section "Approach").

7. Discussion and conclusions

The recorded baseline revealed a relatively high biodiversity of butterflies in farmland of regions in Romania and Spain, including rare and protected species. The suggested monitoring protocol proved to be an adequate and reliable method to monitor these farmland butterfly communities:
(i) the standardised counting approach ensured comparability between sites and countries,
(ii) the applied transect length provided a minimised coefficient of variation
(iii) all time spent in the field was used for monitoring by walking the transects away and back (also increasing sampling intensity)
(iv) a reduced effort scheme of four (five) visits per transect and year reduced monitoring costs,
(v) and additional recorded environmental data could be used for the interpretation of the results.

Power calculations demonstrated that a much lower number of transects would be sufficient to monitor GMO effects on butterflies than previously reported (cf. Lang 2004; Aviron et al. 2009; Lang & Bühler 2012; Lang et al. 2016), although the coefficient of variation for species number and total abundance was similar to the values reported before. However, in this study (i) a matched-pair design was applied, i.e. monitoring the same transects repeatedly, which provided higher statistical power (in contrast to Lang 2004 and Aviron et al. 2009), (ii) the standardised Pollard walk method was used with sufficient transect length (in contrast to Lang 2004 and Aviron et al. 2009), and (iii) the transects were monitored yearly resulting in a high correlation coefficient for butterfly numbers, thus providing higher statistical power (in contrast to Lang & Bühler 2012 and Lang et al. 2016).

For a threshold level of detecting a significant reduction of total abundance of butterflies we suggest a 30 % effect, because (i) threshold effects ≥30 % are already applied for eco-toxicological tests of invertebrate populations (e.g., Barrett et al., 1994; EPPO, 1994), (ii) the International Union for Conservation of Nature (IUCN 2012) classifies a species as “vulnerable” when a population reduction of ≥30 % is observed, and the causes of reduction may not have ceased or may not be reversible (for full description of criteria refer to IUCN 2012), (iii) effects smaller than 30 % may be masked by natural transient fluctuations of butterfly populations, and (iv) detecting effects smaller than 30 % will often require very large sampling efforts.

For a threshold level of detecting a significant loss of species number we suggest a 10 % effect, which is an arbitrary value. We chose this value on the results of the biodiverse regions in Romania (102 species) and Spain (82 species), where we judge a 10 % loss of species to be a matter of environmental concern, i.e. loosing 10 species in Romania and 8 species in Spain.

The necessary sample size to monitor certain GMO effects was relatively similar between countries, subject to outliers. Roughly, about 20 to 30 transects would be needed in a given region to detect effects on species richness (10% loss) and total abundance (30 % reduction), provided the suggested monitoring design is applied. However, higher sampling intensity is required to account for possible outliers and for analysing specific species groups (e.g. red list species), thus a precautionary approach of at least 30 to 60 transects per region are justified.

The monitoring effort in working time follows the required number of transects, and ranges between 22 to 40 working days per year to detect a 10 % species loss, and between 11 – 26 working days per year to detect a 30 % reduction in total abundance, in a given region. Again, monitoring effort needs to be increased if accounting for outliers and analyses of the abundance of specific butterfly species or groups. The transects are sampled yearly in the suggested monitoring design, hence the effort would incur yearly. However, the monitoring effort can be decreased considerably if travelling time to and between transects is minimised (Lang & Bühler 2012). Additional monitoring work has to be accounted for, e.g. organisation, supervision, statistical analyses and reporting.

A prior analysis of expected power should always be among the first steps when planning a monitoring scheme, as this is helpful in providing advice about the required design and sample number to detect given effect sizes (Clark et al. 2006, 2007; Elston et al. 2011; Perry et al. 2003). Designing an effective monitoring scheme for Lepidoptera requires important decisions of how to allocate effort between spatial replicates (no. of transects), temporal replicates (inspection frequency) and sampling area (transect length) (Lang et al. 2016). The results and recommendations presented here showed that a cost-efficient monitoring to detect adverse effects on farmland butterflies is realistic and feasible. Thus, the deliverable 7.2 provides significant guidelines for predictions of power and cost-efficiency of future Lepidoptera GMO monitoring programmes in European farmland.

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Appendix 1

Example of a field data sheet for the recording of butterflies and burnet moths on the transect.

Monitoring protocol — Transect method | **Transect no.** **CAT1** | Sheet 1

Date: **24.04.2014** Time period: 11:47 - 12:38 o'clock (CET) [1] | Location: **Sant Pere de Vilamajor**

Weather: Temperature [2]: 22 °C Wind [0-4 Bft] [3]: 2.5 Proport. sunshine: 100 % | Observer: C Stefanescu

particular incidents *before* survey: [4] | **Direction (away/back)[7]:** **Away**

	Transect sections (each 100 metres long)											
Butterfly species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**A***	Species-specific comments
Crambinae (Snout Moths)												
Lasiommata megera	1											
Pieris rapae		1	2	1	1	4	2	5	3			
Callophrys rubi		1										
Libythea celtis			2	1				1	1			
Pieris brassicae			3	1	1			1				Larval nest on Brassica napus, with L3-L4, section 5.
Lycaena phlaeas			1									
Gonepteryx rhamni				1	2							Males
Gonepteryx sp.				1	1				1			Females
Gonepteryx cleopatra				1								Males
Papilio machaon					1							
Pararge aegeria						1						
Colias crocea							1					
Iphiclides podalirius										1		
Flowering aspect:[5]	1	3	3	3	3	3	1	3	3	3		
Adjacent crop (if any)	Cereal (Avena) + alfalfa	Cereal (Avena) + alfalfa	Brassica napus	Brassica napus	Brassica napus + alfalfa	Brassica napus + alfalfa	Cereal (Avena) + alfalfa	Brassica napus	Brassica napus + cereal	Cereal (wheat?)		*Enter consecutive number and describe on back page.*
Further comments[6]:	Field of rape, increasingly common in the area in the last years.					Alfalfa has been mown.						

* A: Species occurring outside the "5-metre area" (qualitatively only)

1) From 10:00 to 17:00 o'clock (possibly until 18:00 o'clock if weather is extremely dry-warm);

2) At least 13°C if weather is sunny; otherwise, at least 18°C;

3) Bft 0 - 3 allowed; 0 = windless, 1 = wind visible by trails of smoke, 2 = trail of smoke in motion, 3 = leaves and branches constantly in motion, (4) branches constantly in motion, paper whirling up (Bft = Beaufort scale)

4) E.g., intense rain, hail, frost;

5) Categories of flowering aspect for diurnal butterflies of relevant nectar plants: 0 = missing/inferior, 1 = low/poor, 2 = average, 3 = large/high;

6) E.g., habitat or transect use before survey, or current use such as harvest, mowing, etc.

7) Use separate sheets for away and back recording.

Appendix 2

Example of a cover sheet for collecting corresponding data of a transect and the adjacent habitat types.

<table>
<tr><td colspan="2">Transect No.: CAT9</td><td colspan="2">Date: 30 May 2013</td></tr>
<tr><td colspan="4">Field observer: Marina Lee</td></tr>
<tr><td colspan="2" rowspan="2">Name and location of survey site[1]:
Almacelles, about 20 km northwest of Lleida, Spain.</td><td>Exposure:
North-West</td><td>Altitude above sea level:
256 m</td></tr>
<tr><td>Inclination:
0.2%</td><td>Topography: Very flat except sections 5 to 8 that are on a small hill</td></tr>
<tr><td colspan="4">GPS coordinates of transects (start, end):
Start: N41:46:03,93 E0:28:36,60
End: N41:45:42,32 E0:28:37,62</td></tr>
<tr><td colspan="4">General description of habitat/landscape: Intensely cultivated land, the landscape is a mosaic of maize, winter grains and orchards (peach, pear and apple). The area is in the plains but there are small hills on which the original dryland vegetation grows.</td></tr>
<tr><td colspan="4">Composition and structure of field boundaries: Field boundaries are generally very narrow (approx. 0.5 m wide) grassy margins (e. g. Sorghum halepense, Arundo donax, Brachypodium phoenicoides) because they are managed by burning or herbicide applications, at most there are some small shrubs and the occasional tree.</td></tr>
<tr><td colspan="4">Habitat types of the transect sections (according to EUNIS classification)[2]:</td></tr>
<tr><td>Transect section</td><td colspan="3">Habitat types (% per 100 m section)</td></tr>
<tr><td>1</td><td colspan="3">I1.1 (intensive crops) = 65% (70% alfalfa, 30% maize)
G1D4 (orchard) = 35%</td></tr>
<tr><td>2</td><td colspan="3">I1.1 = 100% (50% alfalfa, 50% maize)</td></tr>
<tr><td>3</td><td colspan="3">I1.1 = 100% (50% alfalfa, 50% maize)</td></tr>
<tr><td>4</td><td colspan="3">I1.1 = 100% (100% alfalfa)</td></tr>
<tr><td>5</td><td colspan="3">G1D4 = 100%</td></tr>
<tr><td>6</td><td colspan="3">E1 (dry grasslands) = 100%</td></tr>
<tr><td>7</td><td colspan="3">E1 = 100%</td></tr>
<tr><td>8</td><td colspan="3">E1 = 100%</td></tr>
<tr><td>9</td><td colspan="3">E1 = 55%
G1D4 = 45%</td></tr>
<tr><td>10</td><td colspan="3">E1 = 10%
G1D4 = 90%</td></tr>
</table>

[1] Attach also a print-out of a Google map with plotted transect route, or do a GPS route tracking.
[2] http://eunis.eea.europa.eu/habitats-code-browser.jsp

Appendix 3. EUNIS classification of habitat types (for detailed description: eunis.eea.europa.eu/habitats-code-browser.jsp).

EUNIS classification of habitat types			
B:	Coastal habitats		
	B1:	Coastal dunes and sandy shores	
	B2:	Coastal shingle	
	B3:	Rock cliffs, ledges and shores, including the supralittoral	
C:	Inland surface waters		
	C1:	Surface standing waters	
	C2:	Surface running waters	
	C3:	Littoral zone of inland surface waterbodies	
D:	Mires, bogs, and fens		
	D1:	Raised and blanket bogs	
	D2:	Valley mires, poor fens and transition mires	
	D3:	Aapa, palsa and polygon mires	
	D4 :	Base-rich fens and calcareous spring mires	
	D5 :	Sedge and reedbeds, normally without free-standing water	
	D6 :	Inland saline and brackish marshes and reedbeds	
E:	Grasslands and lands dominated by forbs, mosses or lichens		
	E1 :	Dry grasslands	
	E2 :	Mesic grasslands	
		E2.1:	Permanent mesotrophic pastures and aftermath-grazed meadows
		E2.2:	Low and medium altitude hay meadows
		E2.3:	Mountain hay meadows
		E2.4:	Iberian summer pastures (vallicares)
		E2.5:	Meadows of the steppe zone
		E2.6:	Agriculturally-improved, re-seeded and heavily fertilised grassland, including sports fields and grass lawns
		E2.7:	Unmanaged mesic grassland
		E2.8:	Trampled mesophilous grasslands with annuals
	E3 :	Seasonally wet and wet grasslands	
	E4 :	Alpine and subalpine grasslands	
	E5 :	Woodland fringes and clearings and tall forb stands	
	E6 :	Inland salt steppes	
	E7 :	Sparsely wooded grasslands	
F:	Heathland, scrub and tundra		
	F1 :	Tundra	
	F2 :	Arctic, alpine and subalpine scrub	
	F3 :	Temperate and mediterranean-montane scrub	
	F4:	Temperate shrub heathland	
	F5 :	Maquis, arborescent matorral and thermo-Mediterranean brushes	
	F6 :	Garrigue	
	F7 :	Spiny Mediterranean heaths (phrygana, hedgehog-heaths and related coastal cliff vegetation)	
	F8 :	Thermo-Atlantic xerophytic scrub	
	F9 :	Riverine and fen scrubs	
	FA :	Hedgerows	
	FB :	Shrub plantations	
G: Woodland, forest and other wooded land			
	G1:	Broadleaved deciduous woodland	
	G2:	Broadleaved evergreen woodland	
	G3:	Coniferous woodland	
	G4:	Mixed deciduous and coniferous woodland	
	G5:	Lines of trees, small anthropogenic woodlands, recently felled woodland, early-stage woodland and coppice	
H:	Inland unvegetated or sparsely vegetated habitats		
I:	Regularly or recently cultivated agricultural, horticultural and domestic habitats		
	I1:	Arable land and market gardens	
		I1.1:	Intensive unmixed crops
		I1.2:	Mixed crops of market gardens and horticulture
		I1.3:	Arable land with unmixed crops grown by low-intensity agricultural methods
		I1.4:	Inundated or inundatable croplands, including rice fields
	I.2:	Cultivated areas of gardens and parks	
J:	Constructed, industrial and other artificial habitats		

Appendix 4. Species list and individual abundance of Lepidoptera recorded in **Romania**. Indicated are the protection statuses according to the Annexes of the Habitats Directive, according to the European Red List (Van Swaay et al. 2010) and according to the national Romanian red list (Rakosy 2002). Abbreviations of the red lists are: CR = Critically endangered, EN = Endangered, VU = Vulnerable, NT = Near Threatened, LC = Least Concern. Indicated are also the species of the European Grassland Butterfly Indicator (GS) (Van Swaay et al. 2015) and the common species (C) which occurred in all three study countries (Romania, Spain and Sweden).

No.	Species	Annexes II and IV	European Red List (Europe/EU27)	Red List Romania	Grassland species	Common species	2013	2014	2015
1	Anthocharis cardamines		LC		GS	C		7	5
2	Aporia crataegi		LC				43	54	25
3	Colias chrysotheme		VU	VU					
4	Colias crocea		LC				67	55	6
5	Colias erate		LC	NT/VU					2
6	Colias hyale/alfacariensis		LC				247	182	260
7	Gonepteryx rhamni		LC				4	2	7
8	Leptidea sinapis aggr.		LC				354	83	144
9	Pieris brassicae		LC			C	72	9	18
10	Pieris napi		LC			C	86	58	40
11	Pieris rapae		LC			C	763	203	191
12	Pontia edusa		LC				78	22	19
13	Iphiclides podalirius		LC	NT			56	38	42
14	Papilio machaon		LC	NT			13	9	16
15	Parnassius mnemosyne		NT/LC	NT			1	2	27
16	Apatura ilia		LC	VU					2
17	Apatura iris		LC	VU			1		
18	Aglais urticae		LC			C		2	
19	Araschnia levana		LC			C	2	16	40
20	Argynnis adippe		LC				12	4	4
21	Argynnis aglaia		LC				10	1	1
22	Argynnis paphia		LC			C	100	61	20
23	Boloria dia		LC				35	23	34
24	Boloria euphrosyne		LC				1		
25	Boloria selene		LC					1	
26	Brentis daphne		LC	VU			19	7	26
27	Brentis hecate		LC	VU			11	8	8
28	Euphydryas aurinia	II	LC	EN	GS				2
29	Inachis io		LC			C	65	18	24
30	Issoria lathonia		LC				3	20	11
31	Limenitis populi		LC/NT	VU					
32	Melitaea athalia complex		LC				95	9	27
33	Melitaea britomartis (aurelia)		NT	NT/LC			4	8	1
34	Melitaea cinxia		LC				2	25	14
35	Melitaea didyma		LC				8	3	5
36	Melitaea phoebe		LC				20	12	21
37	Melitaea trivia		LC/NT				2		
38	Neptis sappho		LC	VU			2		11
39	Nymphalis polychloros		LC/VU	VU			1	1	1
40	Nymphalis xanthomelas		LC/NT	CR			1		

41	Polygonia c-album		LC				14	2	12
42	Vanessa atalanta		LC			C	18	4	1
43	Vanessa cardui		LC			C	33	8	16
44	Aphantopus hyperantus		LC				109	190	81
45	Coenonympha arcania		LC				1	3	2
46	Coenonympha glycerion		LC				60	76	67
47	Coenonympha pamphilus		LC		GS	C	644	598	563
48	Erebia medusa		LC				3	1	1
49	Hipparchia cf. fagi		NT	NT/LC					1
50	Lasiommata megera		LC		GS	C	1		4
51	Maniola jurtina		LC		GS	C	879	728	757
52	Melanargia galathea		LC				519	334	412
53	Minois dryas		LC				135	49	63
54	Pararge aegeria		LC					1	3
55	Aricia agestis		LC			C	8		1
56	Aricia eumedon		LC	VU			3		
57	Callophrys rubi		LC				10	1	2
58	Celastrina argiolus		LC				33	8	9
59	Cupido alcetas		LC	EN			1	4	1
60	Cupido argiades		LC				29	65	140
61	Cupido decoloratus		LC	VU			1	9	11
62	Cupido minimus		LC	NT	GS		2	10	18
63	Cupido osiris		LC	VU			1	6	1
64	Glaucopsyche alexis		LC				21	9	22
65	Hamearis lucina		LC				23	7	1
66	Lycaena alciphron		LC/NT	VU			3		
67	Lycaena dispar		LC	VU			1	2	4
68	Lycaena phlaeas		LC		GS	C	10	3	4
69	Lycaena thersamon		LC	VU			5	5	
70	Lycaena tityrus		LC				11	1	8
71	Lycaena virgaureae		LC	NT			1		
72	Maculinea arion	II, IV	EN/EN	NT	GS		3	8	5
73	Plebejus argus		LC				1173	565	495
74	Plebejus argyrognomum		LC					12	28
75	Plebejus idas		LC	NT				74	54
--	Plebejus idas/argyrognomum		LC				105	1	12
76	Polyommatus amandus		LC	EN				2	
77	Polyommatus bellargus		LC		GS		3	7	13
78	Polyommatus coridon		LC		GS		10	7	5
79	Polyommatus daphnis		LC						3
80	Polyommatus icarus		LC		GS	C	72	345	1102
81	Polyommatus semiargus		LC		GS		10	3	6
82	Polyommatus cf. thersites		LC				8	6	23
83	Satyrium pruni		LC	NT			7	3	5
84	Satyrium spini		LC	NT			3	1	1
85	Thecla betulae		LC	NT/VU					1
86	Charcharodus alceae		LC				1	3	3
87	Erynnis tages		LC		GS		38	19	40
88	Hesperia comma		LC				1	3	

89	Heteropterus morpheus	LC	EN				1	
90	Ochlodes sylvanus	LC		GS	C	26	25	34
91	Pyrgus alveus aggr.	LC				1		
92	Pyrgus malvae	LC				18	4	3
93	Thymelicus lineola	LC			C	70	36	120
94	Thymelicus sylvestris	LC				136	21	4
--	Unidentified butterflies					76	226	598
95	Adscita sp.					3		2
96	Jordanita globulariae					2		
97	Rhagades pruni					1		
98	Zygaena carniolica					7	4	4
99	Zygaena filipendulae					6	5	11
100	Zygaena loti					7		
101	Zygaena purpuralis/minos					4		
102	Zygaena viciae					12		
--	Unidentified Zygaenidae					14		3

Appendix 5. Species list and individual abundance of Lepidoptera recorded in **Spain**. Indicated are the protection statuses according to the Annexes of the Habitats Directive, and according to the European Red List (Van Swaay et al. 2010). Abbreviations of the red lists are: CR = Critically endangered, EN = Endangered, VU = Vulnerable, NT = Near Threatened, LC = Least Concern, NA = Not Applicable. Indicated are also the species of the European Grassland Butterfly Indicator (GS) (Van Swaay et al. 2015) and the common species (C) which occurred in all three study countries (Romania, Spain and Sweden).

No.	Transect	European Red List (Europe/EU27)	Annexes II and IV	Grassland species	Common species	2013	2014	2015
1	Anthocharis belia	LC				1		
2	Anthocharis cardamines	LC		GS	C	9	13	12
3	Aporia crataegi	LC				1		1
4	Colias crocea	LC				213	176	181
5	Colias alfacariensis	LC				1		
6	Euchloe crameri	LC				1	15	5
7	Gonepteryx cleopatra	LC				15	41	13
8	Gonepteryx rhamni	LC				11	33	10
9	Leptidea sinapis aggr.	LC				1	7	25
10	Pieris brassicae	LC			C	178	214	34
11	Pieris napi	LC			C	182	71	30
12	Pieris rapae	LC			C	529	722	275
13	Pontia daplidice	LC				38	34	25
14	Iphiclides podalirius	LC				10	18	4
15	Papilio machaon	LC				20	26	29
16	Zerynthia rumina	LC					2	
17	Charaxes jasius	LC				1		2
18	Aglais urticae	LC			C	1	1	
19	Apatura ilia	LC				2		5
20	Araschnia levana	LC			C	1	7	
21	Argynnis pandora	LC						1
23	Argynnis paphia	LC			C	16	4	
22	Boloria dia	LC				8	2	2
24	Brenthis daphne	LC				4		
25	Inachis io	LC			C	11	28	8
26	Issoria lathonia	LC				45	13	11
27	Limenitis reducta	LC				1		3
29	Melitaea cinxia	LC					3	1
28	Melitaea deione	LC				11		
30	Melitaea didyma	LC					2	8
31	Melitaea parthenoides	LC				1		
32	Melitaea phoebe	LC				4	14	7
33	Melitaea trivia	LC/NT					1	2
34	Nymphalis polychloros	LC/VU				2		
35	Polygonia c-album	LC				12	5	3
36	Vanessa atalanta	LC			C	30	14	17
37	Vanessa cardui	LC			C	58	39	127
38	Brintesia circe	LC				14	6	34
39	Coenonympha arcania	LC					3	2
40	Coenonympha dorus	LC				3		
41	Coenonympha pamphilus	LC		GS	C	10	23	15
42	Hipparchia fagi	NT				8	9	2
43	Lasiommata megera	LC		GS	C	127	58	37
44	Maniola jurtina	LC		GS	C	77	98	49

45	Melanargia lachesis	LC			93	278	85
46	Pararge aegeria	LC			113	70	61
47	Pyronia bathseba	LC			13	17	4
48	Pyronia cecilia	LC			19	2	10
49	Pyronia tithonus	LC			49		14
50	Aricia agestis	LC		C	2		2
51	Aricia cramera	LC			6		1
52	Cacyreus marshalli	NA				1	
53	Callophrys rubi	LC			3	3	
54	Celastrina argiolus	LC			9	16	1
55	Cupido argiades	LC			3	22	19
56	Cupido alcetas	LC					1
57	Cupido osiris	LC			1		
58	Glaucopsyche alexis	LC			2		
59	Laeosopis roboris	LC			1		
60	Lampides boeticus	LC			70	9	52
61	Leptotes pirithous	LC			18	158	60
62	Lycaena phlaeas	LC	GS	C	11	22	5
63	Neozephyrus quercus	LC			16	2	4
64	Plebejus argus	LC			4	1	8
65	Polyommatus bellargus	LC	GS		3	3	
66	Polyommatus escheri	LC			3	1	4
67	Polyommatus icarus	LC	GS	C	270	263	291
68	Polyommatus thersites	LC				2	
69	Pseudophilotes panoptes	NT			10	10	3
70	Satyrium acaciae	LC				5	4
71	Satyrium esculi	LC			126	21	30
72	Scolitantides orion	LC/NT					1
73	Charcharodus alceae	LC			67	169	66
74	Carcharodus flocciferus	NT/LC			3	5	
75	Ochlodes sylvanus	LC	GS	C		13	
76	Pyrgus armoricanus	LC			1		2
77	Pyrgus malvoides	LC			6	2	1
78	Spialia sertorius	LC	GS			1	
79	Thymelicus acteon	NT	GS		21	33	14
80	Thymelicus lineola	LC		C	55	87	130
81	Lybithea celtis	LC			20	42	14
--	Unidentified butterflies				130	120	98
82	Zygaenidae spp.					1	

Appendix 6. Species list and individual abundance of Lepidoptera recorded in **Sweden**. Indicated are the protection statuses according to the European Red List (Van Swaay et al. 2010) and according to the national Swedish red list (Ahrné 2015). Abbreviations of the red lists are: CR = Critically endangered, EN = Endangered, VU = Vulnerable, NT = Near Threatened, LC = Least Concern. Indicated are also the species of the European Grassland Butterfly Indicator (GS) (Van Swaay et al. 2015) and the common species (C) which occurred in all three study countries (Romania, Spain and Sweden).

No.	Species	European Red List (Europe/EU27)	Red List Sweden	Grassland species	Common species	2013	2014	2015
1	Anthocharis cardamines	LC		GS	C	2	1	3
2	Gonepteryx rhamni	LC				3		6
3	Pieris brassicae	LC			C	30	26	21
4	Pieris napi	LC			C	309	258	164
5	Pieris rapae	LC			C	238	206	120
6	Aglais urticae	LC			C	236	277	209
7	Apatura iris	LC					1	
8	Araschnia levana	LC			C	2	14	5
9	Argynnis aglaja	LC				2	1	
10	Argynnis paphia	LC			C	4	3	2
11	Boloria selene	LC				3	1	
12	Inachis io	LC			C	19	53	60
13	Issoria lathonia	LC				15	41	1
14	Melitaea athalia complex	LC				3	1	
15	Vanessa atalanta	LC			C	8	24	64
16	Vanessa cardui	LC			C	16	15	11
17	Aphantopus hyperantus	LC				519	359	274
18	Coenonympha pamphilus	LC		GS	C	47	123	50
19	Lasiommata megera	LC		GS	C	9	52	1
20	Maniola jurtina	LC		GS	C	851	636	1230
21	Aricia agestis	LC			C	5	24	8
22	Lycaena hippothoe	LC/NT	NT			1		1
23	Lycaena phlaeas	LC		GS	C	52	28	31
24	Polyommatus amandus	LC					1	
25	Polyommatus icarus	LC		GS	C	64	51	52
26	Satyrium w-album	LC						1
27	Hesperia comma	LC				1		1
28	Ochlodes sylvanus	LC		GS	C	32	30	5
29	Pyrgus armoricanus	LC					4	
30	Thymelicus lineola	LC			C	125	135	219
--	Unidentified butterflies					2	1	1
31	Zygaena filipendulae		NT				1	