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Selection of non-target Lepidoptera species to test *Bt* maize effects in the laboratory: which species and how to breed them?

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Abstract

Bt maize targeting Lepidopteran pests poses potential risks for non-target (NT) butterflies and moths which are addressed in the environmental risk assessment of genetically modified crop plants. For this purpose, eco-toxicological tests are often conducted with specific NT species in the laboratory in order to assess possible adverse effects. As only a limited number of surrogate species can be addressed, the choice of focal species to be tested is an important decision. However, practical and standardised selection procedures have hardly been developed and applied for NT Lepidoptera, so far. Here, we present a transparent and systematic selection process of suitable test species for Germany, involving selection criteria such as exposure to *Bt* maize, habitat range and laboratory maintenance of the species. As a result, we compiled a list of 15 lepidopteran species particularly appropriate for testing the adverse effects of *Bt* maize in the laboratory. In addition, we collected and reviewed published reports for breeding methods of Lepidoptera, which provides essential information on maintaining lab stocks of NT Lepidoptera. The presented selection procedure allows focusing on the relevant test species in a transparent and reproducible way, and supplies the breeding knowledge required to breed and maintain them, which will be of great utility for the future assessment on possible risks of *Bt* maize cultivation to non-target Lepidoptera.

Keywords
breeding, *Bt* maize, ecotoxicity, GMO, Lepidoptera, risk assessment, species selection, test species

Introduction

Transgenic maize is one of the major genetically modified (GM) crops cultivated today (ISAAA 2019). A main application are *Bacillus thuringiensis* (*Bt*) maize events producing insecticidal *Bt* proteins acting against herbivorous pest organisms (Glare and O'Callaghan 2000). The cultivation of *Bt* maize events targeting lepidopteran pests induces potential risks for non-target (NT) butterflies and moths (Lepidoptera). Lepidopteran larvae may ingest insecticidal *Bt* proteins when their larval host plants are dusted by wind-dispersed pollen of *Bt* maize, thus causing additional mortality and/or sublethal adverse effects among larvae (e.g., Zangerl et al. 2001; Dively et al. 2004; Lang and Vojtech 2006; Schuppener et al. 2012). The potential risk that certain *Bt* maize events pose to non-target Lepidoptera is subject to a mandatory pre-release environmental risk assessment (ERA) of GM crops prior to placing on the marked (EC 2001, 2018). The ERA should follow a conceptual step-by-step approach including hazard identification, hazard characterisation and exposure characterisation, and it is described and discussed further in the respective guidelines of the European Food Safety Authority (EFSA 2010a, b). For insect-resistant plants, the testing of non-target organisms (NTO) within the ERA follows an eco-toxicological approach, typically used for testing harmful pesticides and strongly focussing on controlled laboratory tests under standardised conditions (Hilbeck et al. 2008; Lang et al. 2019).

In the EU, the assessment of any GMO is carried out on a case-by-case basis including, amongst other aspects, all receiving environments because a high number of NT species are potentially exposed to GM crops in the field. So far, only a limited, non-representative number of NT Lepidoptera has been studied with regard to the potential hazard of *Bt* maize (Lang and Otto 2010). For example, for the risk assessment of the *Bt* maize event 1507 for non-targets (EFSA 2011; Perry et al. 2012), a species sensitivity distribution was carried out with Lepidoptera that were predominantly pest species (94% of considered species) with a focus on the Noctuidae (56% of considered species), which are known to be rather insensitive to *Bt* (e.g., Pilcher et al. 1997; Binning and Rice 2002). Undoubtedly, there exists a need for more tests with NT Lepidoptera, but because it is not feasible to test every single species for possible adverse effects, a representative subset of NT species referred to as 'focal species' must be selected for the risk assessment (EFSA 2010a). So far, a number of criteria have been proposed for selecting the most suitable species to carry out the ERA for GM plants. For instance, the species' exposure to the respective GM plant, the species' sensitivity to the stressor expressed in the GM crop, the species' occurrence and abundance in the agro-ecosystem, the species' protection status and population vulnerability, the species' representativeness of taxonomical and/or of functional groups, and, considering

practicability, that the species can be bred, kept and tested successfully under laboratory conditions (EFSA 2010a, b; Römbke et al. 2010; Andow et al. 2013; Hilbeck et al. 2014; EFSA 2016). In this respect, general procedures have been proposed, in a stepwise ecologically-based manner, to identify indicator species for testing effects on NTOs and biological diversity (Hilbeck et al. 2011; Andow et al. 2013). However, practical approaches have rarely been conducted by applying operational tools in a systematic, consistent and transparent manner to support the selection of NTO (cf. the case example of Hilbeck et al. 2014).

Several papers have been published dealing with NT Lepidoptera species to be considered for the assessment of possible harmful effects of *Bt* maize. Most of these studies recorded and compiled lists of lepidopteran species that occur near maize fields or in arable land during maize anthesis, and are thus potentially exposed spatially and/or temporally to maize pollen dispersal (Stradling 1999; Losey et al. 2003; Lang 2004; Traxler et al. 2005; Lang and Bühler 2012; Lang et al. 2015; Masetti et al. 2017; Wallis de Vries et al. 2017; Arpaia et al. 2018; Dolezel et al. 2018). Some of these studies also accounted for the occurrence of the respective larval host plants (Stradling 1999; Losey et al. 2003; Wallis de Vries et al. 2017; Arpaia et al. 2018). The maize pollen densities recorded in the environment, i.e. the magnitude of possible *Bt* maize pollen exposure, were considered for the selection of species by Lang et al. (2015) and Arpaia et al. (2018). Some aspects of the species' population vulnerability or protection status were at least mentioned, though not necessarily used for further analysis, by Stradling (1999), Losey et al. (2003), Schmitz et al. (2003), Traxler et al. (2005), Lang et al. (2015), Wallis de Vries et al. (2017), and Dolezel et al. (2018).

To our knowledge, only two studies conducted a comprehensive, systematic and standardised attempt to select and prioritise NT Lepidoptera species for their likelihood to be affected by *Bt* maize, applying selection criteria and resulting in a list of a limited number of focal species to concentrate on in ERA of *Bt* maize (Schmitz et al. 2003; Van Wyk et al. 2007). Van Wyk et al. (2007) applied a ranking matrix based on the methodology described in Andow and Hilbeck (2004) and Hilbeck et al. (2006), focusing on moth species feeding on or closely associated with maize in South Africa, whereas day-active butterflies in adjacent field margins or neighbouring habitats were not considered. Schmitz et al. (2003) analysed a German database of Macro-Lepidoptera (LEPIDAT), developing a risk index and prioritising the species most at risk by *Bt* maize cultivation using a decision tree which accounted for selection criteria such as occurrence in farmland, exposure to maize pollen dispersal or protection status. However, in the study of Schmitz et al. (2003) species not occurring predominantly near maize fields were excluded, thereby neglecting species present but with habitat preferences other than farmland and not considering adjacent habitat types other than field edges. Neither Schmitz et al. (2003) nor Van Wyk et al. (2007) checked for the representativeness of their species' lists, e.g. in terms of representing taxonomic variety, geographical distribution, or different habitat types (cf. Hilbeck et al. 2014). Moreover, the important practical aspect of whether the focal species can be maintained under laboratory conditions was not considered.

Therefore, the objectives of this study were

i. to select a list of "Macro-Lepidoptera" species, which can be considered to be representative in terms of taxonomic diversity, habitat use and body size, and whose larvae appear appropriate as test organisms for studying the effects of *Bt* maize in the laboratory;

ii. to check the feasibility of laboratory breeding of the species by inspecting and listing the literature reports available on breeding Lepidoptera.

Methods

Selection of non-target Lepidoptera species

The aim was to compile a list of potential non-target Lepidoptera species, whose larvae appear generally appropriate as test organisms for studying the effects of *Bt* maize on Lepidoptera in the laboratory. The selection process was carried out by developing and applying a systematic, consistent and transparent selection sequence on the NT Lepidoptera species present in Germany. For this purpose, the national LEPIDAT database of the German Federal Agency for Nature Conservation (BfN) was used to identify suitable species (see also Schmitz et al. 2003), considering geographical distribution, habitats, phenology, host plants, and protection status. The LEPIDAT database refers to Germany with some additions from neighbouring regions and contains 8,670 taxon-specific entries (Pretscher and Klefges 2000).

An initial, pre-selection screening was applied focussing on "Macro-Lepidoptera" for which sufficient biological information was available regarding altitude and habitat type of occurrence as well as for larval feeding periods and feeding type. In this pre-selection step, all "Micro-Lepidoptera" and other species with too little biological information were excluded prior to the actual selection procedure, with one exception. We kept the micro-moth *Plutella xylostella* (Plutellidae), because *P. xylostella* is a well-known and abundant moth in farmland, is very sensitive to *Bt*, and can be bred in the lab easily.

Then, the selection sequence was run with the remaining 1,478 entries for "Macro-Lepidoptera" in order to identify potential test species by applying a step by step process, including expert knowledge in a final step (see below). To begin with, several criteria were checked assessing the exposure of Lepidoptera larvae to *Bt* maize pollen dispersal, i.e. all species that are not exposed were eventually ruled out. Subsequently, the remaining species were prioritised according to the number and type of habitats that they could concurrently occupy. Species were then prioritised according to breeding feasibility in the laboratory by excluding species that cannot be reared well in captivity. The resulting species were then prioritised by conservation status and then, finally, expert knowledge was used to select a representative diversity of species in terms of different habitat types, taxonomic variability, body size distribution and species of protection values.

After the above described pre-selection leaving 1,478 data entries, the subsequent selection process was carried out through the following steps:

Selection Step 1 (excluding unsusceptible species)

Principally, the species-specific susceptibility of lepidopteran larvae to *Bt* maize pollen is a relevant parameter. But as this is still unknown for the majority of the species (cf. Lang and Otto 2010), *Bt* susceptibility was of little discriminatory power and was not applied as a selection factor. The only exception made was that all species belonging to the family Noctuidae (some now in the family Nolidae) were excluded because members of this family were reported to be rather unsusceptible to *Bt* toxins (e.g., Pilcher et al. 1997; Binning and Rice 2002; Pérez-Hedo et al. 2012).

Selection Step 2 (excluding non-resident species)

When selecting NT species for ERA of GM crops, the local receiving environment should be considered; for this reason this study was carried out considering the German maize cultivation areas. Therefore, only species actually occurring in Germany were taken into account (according to LEPIDAT).

Selection Steps 3a–3c (excluding unexposed species)

Species where the larvae are not likely to be exposed to *Bt* maize pollen shedding were excluded (according to the information given in LEPIDAT), i.e.,

a. species occurring exclusively above 700 m a.s.l. where no maize is grown in Germany,
b. species of which larval phenology does not overlap with the maize pollen shedding period, which is roughly from the end of June to the end of August in Germany (Emberlin et al. 1999; Lang et al. 2004; Hofmann et al. 2013),
c. species whose larvae feed endophytically within the host plant, or belowground on roots, thus are not exposed to maize pollen deposition.

Selection Step 4 (excluding species with restricted distribution)

We aimed at selecting widespread species which occur in various different habitats so that the species selected are representative of the range of possible *Bt* maize cultivation environments. So, the specific habitat types as given by LEPIDAT were assigned to each species. Habitat requirements of the species are described in fine detail in the LEPIDAT database, however, for the current approach the species were assigned to the gross habitat classifications: farmland, dryland, wetland, woodland, settlements (if deemed necessary, the specific habitat types can still be ascribed *ex post*). Double entries were possible, e.g., species occurring in two habitat types could be noted for

both habitats. Then, we selected all species which can be found in at least four different habitat types concurrently, including farmland.

Selection Step 5 (excluding species that are difficult to breed in the lab)

Species were selected that could be bred and kept in the laboratory easily according to available knowledge. A literature search was conducted in order to compile information on breeding Lepidoptera families and species, and thus assess the feasibility of breeding each species. Species were defined in three breeding categories: P1 = can be bred in the lab from egg to adult as a stable colony over several generations; P2 = egg laying of adult females is possible in the lab, larvae subsequently can be reared in the lab; P3 = eggs or larvae must be collected in the field but can be kept in the lab thereafter; P4 = keeping and rearing is difficult (no oviposition and problematic keeping in the lab); P5 = unknown. Species that had no record of successful oviposition and rearing in the laboratory were excluded (P3, P4, P5).

Selection Step 6 (prioritising conservation status)

For the risk assessment, protected species are of special concern as they represent a protection goal by EU legislation (EC 1992; EFSA 2010b). Therefore, eco-toxicological testing must identify the sensitivity of protected species and concurrent harm resulting from *Bt* maize effects. The most direct way to estimate this is to include this group in the set of test species. Selection step 6 ensures that protected species are ranked high and are included in the final set of test organisms provided they could be bred in the lab well.

Step 7a to 7c (ensuring a representative variety of species)

In the following steps, the species selection was further fine-tuned with regard to a representative distribution of:

a. taxonomic variety (covering different Lepidoptera super-families);
b. body sizes (covering a range of different larval sizes); and
c. further factors depending on the respective receiving requirement, i.e. the area where the GM crop is cultivated (e.g., geographical distribution, abundance in the field, ecological significance; cf. Hilbeck et al. 2014). This was done according to our own expert judgement in order to warrant a diverse, representative list of species, including protected species if feasible.

Breeding and rearing European Lepidoptera

A screening for existing breeding methods of Lepidoptera was carried out through a literature search, supplemented with expert interviews. A general internet search using google and google scholar did not produce many valuable results, although some non-academic information exists on different internet sources. Relevant literature was

Table 1. Superfamilies and families of Lepidoptera screened for breeding information (taxonomy according to www.fauna-eu.org). Number of European species according to Rennwald and Rodeland (2019).

Superfamily	Family (species number in Europe)
Bombycoidea	Brahmaeidae (7), Endromidae (2), Saturniidae (11), Sphingidae (42)
Cossoidea	Brachodidae (15), Castniidae (1), Cossidae (37), Sesiidae (117)
Drepanoidea	Cimeliidae (3), Drepanidae (22)
Geometroidea	Geometridae (1,092), Uraniidae (1)
Hepialoidea	Hepialidae (18)
Lasiocampoidea	Lasiocampidae (48)
Noctuoidea	Erebidae (372), Euteliidae (2), Noctuidae (1,306), Nolidae (48), Notodontidae (59)
Papilionoidea	Hesperiidae (48), Lycaenidae (151), Nymphalidae (261), Papilionidae (16), Pieridae (61), Riodinidae (1)
Zygaenoidea	Epipyropidae (2), Heterogynidae (13), Limacodidae (5), Somabrachyidae (1), Zygaenidae (68)

mostly retrieved from general biological databases such as BIOSIS (http://isiknowledge.com/biosis), but also from two specific databases (www.entomologische-literatur.de; www.zobodat.at). "Entomologische Literatur" is a private database on publications on Lepidoptera from Germany, whereas "Zobodat" is the publication database of the Biologiezentrum Linz, Austria. These two more specific databases offer access to publications from the beginning and the middle of the 20th century, mostly in the German language. Many of the publications cover "traditional breeding" defined as breeding without controlled (microclimatic) conditions. The most valuable search strings for the German/Austrian databases were <Zucht> (German for breeding/rearing) and family names. Searching BIOSIS was rendered most efficient by using the Lepidoptera family name together with <laboratory rearing> or <artificial diet>.

The search on breeding focused on the superfamilies and families presented in Table 1, thus excluding largely the "Micro-Lepidoptera (see also Selection of non-target Lepidoptera species). Geographically, the search was restricted to Europe. The first screening yielded an enormous number of publications, especially for certain families, which made it impossible to consider all breeding references ever published. Therefore, we followed the subsequent strategy: after we had collected sufficient information for the information-rich families, we then focused the further search on those families, where breeding information was scarce, so far.

All publications selected using the above procedure were listed in a table (see Suppl. material 1) and information about the type of breeding is provided. In addition, more details are presented on the content of the publications, including information on which species were bred successfully on an artificial diet. Breeding on an artificial diet was considered particularly relevant, because it allows standardization and reproducibility of laboratory trials.

Results

Selection of non-target species

Pre-selection: overall, the LEPIDAT database contained 8,670 entries for Lepidoptera taxonomic units (genera, species, sub-species). Excluding all taxa for which the

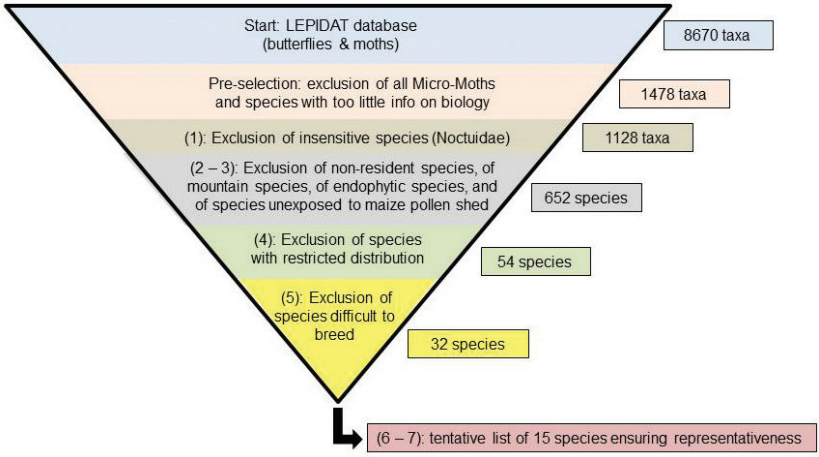

Figure 1. Schematic presentation of the selection process to choose focal Lepidoptera species for the testing of *Bt* maize effects in the laboratory.

database did not contain the information required by the following selection steps resulted in 1,478 taxa for selection step 1 (Fig. 1).

Selection step 1 (excluding unsusceptible species): species known to be insensitive to *Bt* were excluded, in this case all 350 listed species belonging to the family Noctuidae (some now in the family Nolidae), leaving 1,128 taxa (Fig. 1).

Selection step 2 (excluding non-resident species) and step 3 (excluding unexposed species): of the remainder, 476 taxa were eliminated when applying the exclusion criteria of steps 2 and 3, i.e. species unlikely to be exposed to *Bt* maize in Germany were excluded; either because the species are not native (= step 2), or because they are not exposed due to their altitudinal distribution, phenology or feeding habits (= steps 3a–3c). In consequence, a total of 652 Lepidoptera species remained as potentially adequate test species (Fig. 1). This selection encompassed species including butterflies and moths: 60 Nymphalidae, 42 Lycaenidae, 17 Hesperiidae, 12 Pieridae, 2 Papilionidae, 304 Geometridae, 43 Arctiidae, 35 Psychidae, 31 Notodontidae, 19 Zygaenidae, 15 Drepanidae, 15 Nolidae, 14 Lasiocampidae, 14 Sphingidae, 12 Lymantriidae, 4 Cossidae, 4 Hepialidae, 4 Cossidae, 2 Limacodidae, 1 Gracillariidae, 2 Saturniidae, 1 Endromidae, 1 Lemoniidae, 1 Thyrididae and 1 Plutellidae.

Selection step 4 (excluding species with restricted distribution): assigning habitat types to the remaining 652 species produced a list of 479 "woodland species", 304 "farmland species", 271 "dryland species", 207 "wetland species", and 161 "settlement species" (multiple nominations of species to habitat categories were possible). From this pool, we selected all species which can be found in four different habitat types concurrently including farmland, as this covers widespread species representative for several different habitat types. This resulted in a list of 54 species (Fig. 1).

Selection step 5 (excluding species that are difficult to breed in the lab): here, we assessed the species' suitability for lab culturing, i.e. whether the species are relatively

easy to breed. All species were selected that oviposit in the lab and/or can be reared as larvae indoors (P1 and P2) according to the defined breeding categories. This resulted in a list of 32 species (Table 2).

Selection steps 6 (prioritising conservation status) – 7 (ensuring a representative variety of species): All 32 species (Table 2) obtained in the previous selection step are potentially suitable for testing *Bt* maize pollen effects on lepidopteran larvae in the laboratory. As a final outcome of our selection, we compiled a tentative list of 15 test species (species marked in bold in Table 2) in order to contain at least one species of protection value (step 6); and include species of higher abundance in farmland belonging to different super-families (taxonomic variety) with larvae of different body sizes (step 7).

Table 2. List of 32 Lepidoptera species suitable for laboratory experiments to test the adverse effects of *Bt* maize. A tentative list of 15 focal species is marked in bold. Breeding categories: "P1" = can be bred in the lab from egg to adult as a stable colony over several generations; "P2" = egg laying of adult females possible in lab, larvae can be reared on host plant leaves or artificial diet; see methods for details. Red list classifications: "EN" = endangered, "VU" = vulnerable", "NT" = near threatened, "LC" = least concern (BfN 2011).

Species	Family	Red List Germany	Breeding category
Arctia caja	**Arctiidae**	**NT**	**P1**
Diacrisia sannio	**Arctiidae**	**LC**	**P1 to P2**
Diaphora mendica	Arctiidae	LC	P1 to P2
Euplagia quadripunctaria	Arctiidae	LC	P1 to P2
Parasemia plantaginis	Arctiidae	NT	P1
Ematurga atomaria	**Geometridae**	**LC**	**P1 to P2**
Peribatodes rhomboidaria	Geometridae	LC	P1 to P2
Scopula immutata	Geometridae	LC	potentially P1/P2
Pyrgus malvae	**Hesperiidae**	**NT**	**P1**
Lycaena tityrus	Lycaenidae	LC	P1
Lycaena virgaureae	Lycaenidae	NT	P1 to P2
Aglais io	**Nymphalidae**	**LC**	**P1**
Aglais urticae	**Nymphalidae**	**LC**	**P1**
Aphantopus hyperantus	Nymphalidae	LC	P1
Argynnis adippe	**Nymphalidae**	**VU**	**P1**
Argynnis aglaja	Nymphalidae	NT	P1
Boloria selene	Nymphalidae	NT	P1
Coenonympha pamphilus	**Nymphalidae**	**LC**	**P1**
Erebia medusa	Nymphalidae	NT	P1 to P2
Euphydryas aurinia	Nymphalidae	EN	P1
Hipparchia semele	Nymphalidae	VU	P1
Maniola jurtina	**Nymphalidae**	**LC**	**P1**
Melitaea athalia	Nymphalidae	VU	P1 to P2
Minois dryas	Nymphalidae	EN	P1 to P2
Anthocharis cardamines	Pieridae	LC	P1
Pieris brassicae	**Pieridae**	**LC**	**P1**
Pieris napi	**Pieridae**	**LC**	**P1**
Pieris rapae	**Pieridae**	**LC**	**P1**
Hamearis lucina	Riodinidae	VU	P1 to P2
Deilephila elpenor	**Sphingidae**	**LC**	**P1**
Hyles galii	**Sphingidae**	**LC**	**P1 to P2**
Plutella xylostella	**Plutellidae**	**LC**	**P1**

Breeding and rearing European Lepidoptera

General information

The literature search for breeding and rearing European Lepidoptera resulted in a list of 548 publications including a number of handbooks and reviews, which are all compiled and listed in Suppl. material 1. Details about the contents of the publications are presented concerning which species (groups) are treated as well as on the general subject of the references, and the specific contents of the studies are described in more detail such as specific conditions required for successful rearing of larvae (Suppl. material 1, 2). Early descriptions are more than 100 years old (e.g. Holtheuer 1908; Lutz 1904), and mainly address amateur lepidopterists, who wish to keep collected caterpillars at home. In general, Fritzer (2005) and Gleichauf (1968) describe rearing caterpillars and pupae only, while all stages are treated by Aue (1928), Müller (1986, 1987a, b), Weidemann (1982–1984), and Wyniger (1974). The methodology of hand-pairing of imagines is described by Clarke and Sheppard (1956), Müller (1987b), Platt (1969) and Weidemann (1982–1984). An extensive, up-dated handbook on traditional breeding of European Lepidoptera was published by Friedrich (1983; English version published in 1986). The handbooks of Singh and Moore (1985) and Wyniger (1974) additionally treat breeding on an artificial diet. Checking literature on larval ecology can also provide valuable information on the specific host plants used by the species (e.g. Carter and Hargreaves 1986; Bräu et al. 2013; Reinhardt et al. 2020).

In the laboratory, Lepidoptera are often reared in the traditional way, i.e. on natural host plants under room conditions without control of the microclimate. Keeping larvae on their natural host plants is also the recommended approach for testing *Bt* maize effects (Lang et al. 2019). Commonly, only caterpillars are reared to adults, because to maintain a colony in the lab would require covering the whole life cycle from eggs to pairing adults. Whatever the approach, certain rearing basics have to be taken into account which we compiled in Table 3. Hygienic conditions are of utmost importance with a regular change of food and container as well as good ventilation to prevent water condensation leading to mould formation. For mass rearing, eggs or pupae are often disinfected in order to reduce incidence of disease (e.g., Bathon and Gröner 1977). If breeding over several generations is wanted, measures to maintain fitness and avoid inbreeding are needed (Müller and Wintermann 1985; Bryant et al. 1999), and sometimes it may be necessary to supplement the colony with fresh individuals from the field. All containers and handling equipment should routinely be disinfected (Morton 1979), e.g. by UV light (Fiedler, pers. comm.). In some cases it may also be necessary to treat the food plants to remove infectious agents or parasitoid eggs present on the natural host plants (Stefanescu, pers. comm.).

Breeding on artificial diets

For testing *Bt* maize pollen effects, Lang et al. (2019) recommend to use natural host plants for rearing lepidopteran larvae as this mimics the natural situation best, thus

Table 3. Background information required when breeding Lepidoptera.

Factor	Larvae	Adults
Population origin	Is it possible to obtain caterpillars or other immatures from a laboratory population? How can immatures be obtained from the field?	How can the adults be obtained (light trapping, netting…)?
Food	Which plants and plant parts serve as food? Is the food permanently available? What particular requirements do the food plants need to meet (water, nutrition)? Can caterpillars be reared on artificial diets (see below)?	Do adults require feeding in order to mate/oviposit? Is forced feeding possible or necessary? What type of food do adults require? Is food permanently available (i.e. flowers)? Which plants or substrates are needed for oviposition?
Climatic conditions	What is the temperature range for an optimal development? What is the relative humidity needed for development and to minimise disease incidence? Which day/night cycle is needed? Which conditions should be avoided to prevent dormancy or diapause?	What are conditions for adults to mate and oviposit (photoperiod, sunlight, temperature)?
Rearing containers	What are the required dimensions or other characteristics of the rearing containers?	What requirements do adults need for eclosion? What dimensions are necessary in order to achieve mating?
Population density	How many caterpillars can be reared together (competition, infections)?	How many adults (female:male ratios) should be placed together to achieve mating?
Life cycle	What are the development times for the different life stages and at what temperatures?	Do adults show dormancy or diapause and what are the triggers?
Handling	Is there any special consideration regarding handling (i.e. larval stress, damaging moulting stages)? How many larvae can be reared at a time by the workforce (number of working hours available)?	How often will it be possible to obtain large numbers of eggs?
Disease/ predator control	What are the most frequent diseases and how can they be prevented or reduced, can eggs or pupae be disinfected and how? Is it necessary to wash/disinfect the host plant?	Is it necessary to take any particular measures for adult feeding or egg laying, e.g. washing or disinfecting wildflowers or oviposition substrate?
Others	Are there special requirements for the larvae to pupate (e.g. substrate)? Are there any particular other aspects of the species that need to be considered (e.g. myrmecophily)?	Is hand-pairing necessary and feasible? Is it possible to preserve adults, pupae or eggs in the refrigerator, for how long?

increasing the ecological relevance and validity of the test results. Nevertheless, using artificial diets might be reasonable in some cases, e.g. in order to standardise experimental conditions or to quickly screen a large number of different species. Here, general information on using artificial diets is presented, while more detailed descriptions can be found in Suppl. material 1, 2. Artificial diets for rearing lepidopteran larvae were developed in the 1960s (Grisdale 1963, 1973; House et al. 1971; Singh 1977), and since then they have been improved and diversified. Artificial diets usually consist of flour (often from legumes or cereals), proteins, yeast, cholesterol, vitamins and minerals. Agar is used as binding material, sometimes antibiotics and other preservatives are added. If a phagostimulant is needed, dried and ground food plants and/or sucrose may be mixed into the diet (Morton 1979). When rearing lepidopteran larvae on artificial diets, specific containers have to be used (Singh and Surrey 1980; Hansen and Zethner 1979; Skovmand and Christoffersen 1994; Davis et al. 1990).

Several publications exist that give an overview of different artificial diets and/or list successfully reared species, e.g. Gardiner (1978), Hansen and Zethner (1979), Khalaf (1979), Singh (1980, 1983). Recipes of artificial diets repeatedly used are given by Bergomaz and Boppré (1986) and McMorran (1965) with modifications by

Grisdale (1973), Singh and Moore (1985) and Wyniger (1974). Fischer et al. (1991) used the recipe of Bergomaz and Boppré (1986) and published their experiences and listed successfully reared species. An overview of species that were successfully reared on the "McMorran diet" (McMorran 1965, Grisdale 1973) are given by Hervet et al. (2016). Reinecke (1985) considers in detail the different types of diet, nutritional components, gels and bulking agents, water, feeding inducers, diet stabilization and antimicrobials and physical aspects. Vanderzant (1974) and Bell et al. (1978) also provide overviews.

Depending on the natural feeding habits, artificial diets must be presented in different ways to mimic natural conditions (Gardiner 1978; Morton 1979). Nevertheless, the acceptance of artificial diets by caterpillars is not always easily obtained. Especially first instar caterpillars are often reluctant to accept artificial diets (e.g. Fiedler pers. comm., Fischer et al. 1991, Hervet et al. 2016). If caterpillars had contact with food plants, a shift to an artificial diet may not be successful or cause high mortality (Fischer et al. 1991; Morton 1979). The artificial diet must be exchanged regularly to avoid desiccation of the diet and/or mould formation (Fischer et al. 1991, Fiedler pers. comm., Morton 1979). Some recipes add formalin to avoid mould formation (e.g. Bergomaz and Boppre 1986; Morton 1979). It is advised not to allow for pupation on/in the artificial diet (Gardiner 1978).

Discussion

Here we present a list of potential, non-target Lepidoptera species for assessing the effects of *Bt* maize pollen on lepidopteran larvae in the laboratory, and develop a systematic selection process allowing to identify the suitable test species in a transparent manner. A number of criteria have been proposed for selecting adequate non-target species of various taxonomic groups to assess the potential environmental risks of cultivating GM plants (e.g., EFSA 2010b). In the case of lepidopteran-specific toxicants expressed by *Bt* maize, the direct harm to Lepidoptera is known and documented (e.g., Lang and Otto 2010), but requires quantification on a number of species. Variation of lepidopteran species in Europe is very high in terms of sensitivity to *Bt*, in terms of temporal and spatial exposure to maize cultivation, and in terms of vulnerability of their populations, hence testing only a few species for a specific *Bt* toxin is not sufficient (Lang and Otto 2010). On the other hand, it is not feasible to test all exposed Lepidoptera. Therefore, when choosing a limited number of focal species for toxicological tests, it is important that the selected species are sufficiently representative to act as proxies of the remaining, ignored species. The presented selection method fulfils this need in generating an appropriate and representative set of candidate test species. The species selection was done by subsequently excluding less suitable species following a systematic sequence of criteria, resulting in a list of 32 potential, non-target Lepidoptera test species. From those, we chose an array of 15 focus species in order to cover the range of diversity encountered across Central Europe, using the selection steps 4, 6

and 7 to create representativeness in terms of widespread species occurring in various habitat types, encompassing a wide geographical range and a large number of different environments, and covering taxonomic variety, protection values and morphological features such as larval body size.

Importantly, a crucial selection criterion was step 5, the possibility to breed the species in the laboratory, which is of relevance when keeping specimens for testing purposes and establishing lab cultures of test organisms (Hilbeck et al. 2014). In our research on breeding Lepidoptera, we considered 9 super-families with 30 families, representing the so-called "Macro-Lepidoptera". For all families that have larger numbers of species, reports on breeding for several species exist. In most families, some species were also reared on an artificial diet, indicating that good procedures exist to breed these species in the laboratory without the need to cultivate the respective host plants. Even for some families consisting of very few species in Europe, information on breeding could be retrieved (see Suppl. material 1, 2). Clearly, this allows to select a good taxonomic range of species across "(Macro-)Lepidoptera", e.g. the species of our selection process in Table 2 represent 4 of these 9 super-families. This means that there is no general lack of breeding information impeding lab rearing of test species, with the *caveat* that for specific cases and circumstances establishment of lab cultures can still pose problems, and in such cases it may take time to develop a workable rearing method to obtain sufficient healthy larvae for experiments.

In contrast to the initial systematic selection criteria (steps 1–5), the last selection steps 6 and 7 were done according to expert assessment, as was proposed by other published selection protocols (Hilbeck et al. 2014). Such an expert judgement serves as a cross check in order to review if relevant species were missed by the operational selection process. Our selection of widespread species occurring in farmland and other habitat types (step 4) favours generalist and mobile species rather than more specialised, stationary species living in habitats other than farmland. However, protected species are of special conservation concern (EFSA 2010a, b; 2015), and non-farmland habitats hosting endangered species may also receive wind-dispersed *Bt* maize pollen (Lang et al. 2015). Thus, expert choice in step 6 ensured the consideration of these protected species, which may well vary with the bio-geographical region (Dolezel et al. 2018). In step 7, further aspects were taken into account to ensure a representative variety of species. For example, taxonomic variety covering a range of different Lepidoptera super-families is important, because different species (groups) may differ e.g. in their sensitivity to *Bt* (Peacock et al. 1998; Wolt et al. 2005). Species sensitivity distributions (SSD) for toxicological effects rely on sets of representative test species (Posthuma et al. 2002). So far, only one SSD was calculated for Lepidoptera and *Bt* maize (EFSA 2011; Perry et al. 2012), including mainly pest species of only a few Lepidoptera super-families with a focus on the often *Bt*-insensitive Noctuidae. Considering a wider taxonomic range of NT Lepidoptera species would improve the reliability of SSD results. We consider larval body size to be an additional relevant aspect for species selection, because smaller larvae tend to be more sensitive to the effects of *Bt* pollen uptake (Felke and Langenbruch 2005; Wolt et al. 2005).

In our study we used LEPIDAT, a database compiled by the BfN (Pretscher and Klefges 2000; Schmitz et al. 2003). In the meantime, LEPIDAT has been replaced by the web application "Schmetterlinge Deutschlands" (https://www.schmetterlinge-d.de/)." Species excluded or not considered by our approach may in fact be suitable and valuable test species, e.g. some Micro-Lepidoptera, which were discounted due to the lack of available biological information. Other Lepidoptera databases could be valuable and basic sources for the environmental risk assessment of transgenic crops, too, and existing ones should be assessed for their respective suitability, and adapted if required (e.g. the eBMS database).

It has to be noted that our selection procedure resulted in the general identification of representative species suitable for laboratory experiments and does not aim to recognise a specific hazard to certain species in advance. In fact, the potential hazard has to be studied in the subsequent lab trials, which is also why the still mostly unknown susceptibility of single lepidopteran species to *Bt* maize pollen was not a selection criterion for choosing the test species in our study. Any selection procedure for test species must identify and take into account the species of the local, corresponding environment, i.e. the species that would be exposed in the area where the *Bt* maize is to be grown. If applying our selection process to regions in Europe other than Germany, the conditions determining the magnitude of the exposure of certain lepidopteran species to *Bt* maize will differ, and the selection process should be adapted accordingly, e.g. with respect to the altitude ranges of maize cultivation or times of maize pollen shedding.

Conclusions

In view of the thousands of lepidopteran species in Europe it is indispensable to focus on only a limited range of species for the assessment of adverse effects of *Bt* maize on lepidopteran larvae. On the other hand, it is also paramount to consider a sufficient number of species to assure a reasonable representativeness of the tested species group. Our stepwise selection procedure provides a systematic, transparent and generic approach to create a representative list of NT Lepidoptera for *Bt* maize testing. This is a major achievement as a standardised protocol on how to select the relevant indicator species did not exist up to now. The selection process is generic in the sense that it can be simply adapted to other locations as well as to specific requirements and objectives. In our case, the resulting species list is highly representative and exceeds the so far limited range of studied test species for the risk assessment of *Bt* maize in Central Europe. Breeding feasibility is of crucial importance for maintaining laboratory cultures of test species, on which there is a large body of literature. For all species-rich Lepidoptera families many reports on breeding for several species exist; this often includes rearing on an artificial diet. In other words, laboratory testing of NT Lepidoptera species appears feasible for a very broad taxonomic range, which is summarised here for the first time. Thus, any risk assessment involving the breeding of butterfly and moth larvae will benefit from the information presented. In particular, NT testing of Lepidoptera in Europe for the assessment of *Bt* maize will greatly profit from following the reported approach, information and results.

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Supplementary material 1

Reference list: breeding and rearing Lepidoptera

Authors: Andreas Lang, Matthias Dolek, Marina S. Lee, Anja Freese-Hager, Mathias Otto

Data type: Literature list (Excel table)

Explanation note: List of publications reporting on breeding Lepidoptera with short description of study contents.

Link: https://doi.org/10.3897/biorisk.15.59823.suppl1

Supplementary material 2

Review breeding information

Authors: Andreas Lang, Matthias Dolek, Marina S. Lee, Anja Freese-Hager, Mathias Otto

Data type: Breeding info (pdf)

Explanation note: Short review of how to breed Lepidoptera of various families in the laboratory.

Link: https://doi.org/10.3897/biorisk.15.59823.suppl2