Estimating acute mortality of Lepidoptera caused by the cultivation of insect-resistant *Bt* maize – The *LepiX* model

Lorenz Fahse[a,*], Phillip Papastefanou[a,b], Mathias Otto[c]

[a] *University of Koblenz-Landau, Institute for Environmental Sciences, Fortstraße 7, 76829 Landau, Germany*
[b] *Land Surface-Atmosphere Interactions, Department of Ecology and Ecosystem Management, Technical University of Munich, Hans-Carl-von-Carlowitz-Platz 2, 85354 Freising, Germany*
[c] *Federal Agency for Nature Conservation (BfN), Konstantinstraße 110, 53179 Bonn, Germany*

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ABSTRACT

The cultivation of *Bt* maize, genetically modified to be resistant to insect pests, has led to intense scientific and political debate about its possible adverse impacts on biodiversity. To better address this question we developed an individual-based simulation model (*LepiX*). *LepiX* considers the temporal dynamics of maize pollen shedding and larval phenology, and pollen deposition on host plants related to distance from the maize field, in order to estimate mortality of lepidopteran larvae exposed to toxic *Bt* pollen. We employed a refined exposure analysis, comparison to previous approaches, using recent evidence on leaf pollen deposition and accounting for the spatial heterogeneity of pollen on leaves. Moreover, we used a stochastic approach, considering literature data on a minimum dataset for butterfly biology in combination with historic data on temporal pollen deposition to predict the coincidence between larval phenology and pollen deposition. Since conservation management actions may act at the level of the individual for protected species, *LepiX*, as an individual based spatially explicit model, is suited to assist both risk assessment and management measures based on threshold mortalities. We tested our model using *Inachis io* (Lepidoptera: Nymphalidae) as butterfly species and the cultivation of insect resistant MON810 maize. In accordance to predictions based on other models we identified mortality risks of *I. io* larvae for the second larval generation. An analysis of the sensitivity of input parameters stressed the importance of both the slope and the LC_{50} value of the dose-response curve as well as the earliest day of larval hatching. Using different published data to characterize the dose-response of MON810 pollen to *I. io* we revealed consequences due to uncertainties in ecotoxicological parameters and thus highlight the importance of key biological parameters for reliable estimates of effects, and decision making (e.g. isolation distances) in risk assessment.

1. Introduction

Genetically modified (GM) crops are cultivated worldwide, and most of these crops are resistant to herbicides, insects or both (Parisi et al., 2016). The commercial use and release of GM crops in the environment is regulated and only granted after the risks for human health and the environment have been assessed (EC, 2001).

Insect resistance is most commonly implemented by transferring genes originating from the soil bacterium *Bacillus thuringiensis* into the crop which will express insecticidal Cry or Vip proteins in the plant tissues (Glare and O'Callaghan, 2000). A number of *Bt* maize plants target Lepidoptera, such as the European corn borer (Lepidoptera: Crambidae, *Ostrinia nubilalis*) and the Mediterranean corn borer (Lepidoptera: Noctuidae, *Sesamia nonagrioides*). However, as *Bt* proteins do

not act species specific (van Frankenhuyzen, 2009, 2013), effects of *Bt* crops on non-target organisms, e.g. non-target butterfly larvae, have to be considered in the risk assessment (Andow and Hilbeck, 2004; Andow and Zwahlen, 2006; Hilbeck et al., 2011; Lang and Otto, 2010; Marvier, 2001; O'Callaghan et al., 2005; Romeis et al., 2008; Wolfenbarger and Phifer, 2000).

In most *Bt* maize, the *Bt* proteins are expressed in all plant tissues, including pollen. As maize is wind pollinated and produces large amounts of pollen (Eastham and Sweet, 2002), habitats adjacent to *Bt* maize cultivation fields will be exposed to pollen containing *Bt* proteins (Hofmann et al., 2010), and off-field effects have to be considered in the risk assessment. Maize flowering and the larval phenology of many butterfly species overlap; therefore, non-target butterflies in Europe are likely at risk (Lang et al., 2015; Musche et al., 2009; Schmitz et al.,

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* Corresponding author.
 E-mail address: lfahse@uni-landau.de (L. Fahse).

2003; Traxler and Gaugg, 2005). This risk will depend on a variety of factors, such as the degree of temporal overlap in phenologies, amount of pollen to be expected on host plants, toxin concentration in pollen and sensitivity of the non-target species.

Risk assessment prior to GMO authorization requires substantial data and analytical tools. In 2009, the European Food Safety Authority (EFSA) deployed a first mathematical simulation model to predict the consequences of cultivation of *Bt* maize on non-target Lepidoptera (EFSA, 2009; Perry et al., 2010). This model has been developed further to take new aspects and data for the assessment into account (EFSA, 2011a,b, 2012, 2015). In addition, Holst et al. (2013a,b) estimated the mortality of the Peacock butterfly *Inachis io* caused by *Bt* maize in a simulation model of the butterfly's annual life cycle over-laid with the phenology of maize pollen deposition on the leaves of the food plant *Urtica dioica*. However, the model has not been used in the context of regulatory purposes and did not include spatial aspects, such as the distance of larvae and host plants to the maize field.

Here, we present a new simulation model (*LepiX* 1.0) to estimate off-field effects of *Bt* maize cultivation on non-target butterfly species. Our model is designed to simulate the temporal overlap of maize flowering and larval phenology, pollen deposition on host plants depending on the distance to the maize field, and the effect of *Bt* pollen exposure on the mortality of butterfly larvae. Because we can obtain information on both individual larvae and populations *LepiX* is especially suited to analyse effects on rare, endangered and/or protected Lepidoptera as these may be protected on the level of the individuum and/or population. The exposure module of our model uses the so far most comprehensive set of field data from research projects dealing with maize pollen deposition, which were initiated by the German Federal Agency for Nature Conservation in collaboration with German federal states to improve the assessment of *Bt* maize exposure on non-target organisms (Hofmann et al., 2009, 2011, 2013, 2014, 2016).

2. The *LepiX* model

At first, we specify the purpose of the model (Section 2.1) and give a general overview of its structure (Section 2.2.1) before all submodels are described in detail (Section 2.2.2).

2.1. Purpose

The simulation model *LepiX* (*Lepi*doptera and ecotoxicology) 1.0 is a tool to estimate mortality probabilities for non-target insect species affected by *Bt* maize, dependent on the distance from the maize field. The information obtained assists the risk assessment and risk management of genetically engineered plants that express *Bt* proteins as insecticidal components.

2.2. Model description

In principle, *LepiX* is a generic model that can be parametrized for any species or group of butterflies or moth and for any geographical location and for any GM crop variety. As a case study we used the Bt-maize event MON810 and the non-target species *Inachis io* at two locations in Germany (Bad Hersfeld and Rheinfelden), where it is known to be univoltine and bivoltine, respectively (see Section 3).

2.2.1. General structure and design

Species protected under e.g. the German Federal Species Protection Regulations (Bundesartenschutzverordnung; BArtSchV) are protected not only as populations but also as individuals. Therefore, a model for risk assessment of individuals is desirable and motivates an individual-based approach (Grimm and Railsback, 2005). *LepiX* is discrete in time and simulates a time period of one year with a time step of one day. For each year, both a specific temporal pattern of pollen shedding during the flowering period and mean daily temperatures are randomly chosen

from historical field data. These temporal patterns serve as input data for the simulation of butterfly phenology and exposure. The daily development of larvae depends on the sums of daily temperatures (weather). The risk of mortality per day depends on the amount of pollen one single larva is exposed to daily. The quantitative exposure is not equal for all larvae; therefore, we included spatial variation of the deposition on host plants according to the data of Hofmann et al. (2016). This leads to an individual pollen exposure for each larva in the model.

The *LepiX* model simulates the development and exposure of 1000 larvae during one year. This simulation was repeated 1000 times with different randomly chosen temporal patterns for flowering periods (pollen shedding) and daily temperatures (weather). In the following, we refer to these 1000 simulated populations as 1000 "simulation runs" and denote the ensemble of the 1000 simulated populations as one "scenario" (i.e. for one fixed model parameter setting).

The distances between the maize field and the location of maize pollen deposition were taken into account by using long-term monitoring data of fields which were cultivated under common agricultural conditions (Hofmann et al., 2014). In this way, the *LepiX* model is in substance spatially implicit, i.e. the exact positions of the larvae in space or on the host plants are not considered.

The *LepiX* model consists of five modules (Fig. 1). The weather module provides the daily temperatures of one year and passes this information to the individual module, where the individual larval periods are simulated. The pollen deposition module provides the amount of *Bt* maize pollen on the host-plant leaves at any distance from the maize field and for each day of the year. The exposure module simulates the relevant exposure to pollen of each larva. The individual exposure rates finally result in the individual risks of mortality (mortality module).

In the *LepiX* model, the individuals do not interact, and no reproduction or resource limitation is considered. Stochasticity plays a major role in all sub-models (for details, see Section 2.2.2). Especially the start of the larval stage and the daily variation in consumed pollen are drawn from probability distributions, which also stochastically affect individual traits, e.g. the development time of a larva.

The model is implemented in C# following a purely object-orientated approach and agent-based modelling design patterns (Grimm and Railsback, 2005) using *R* for statistical post-process analysis (R Core Team, 2016).
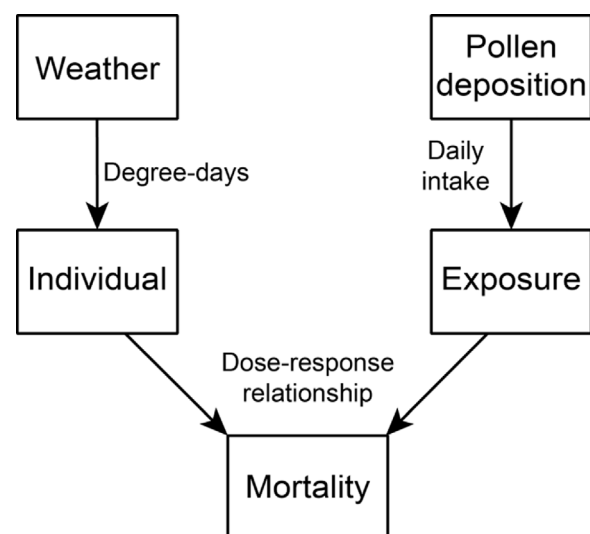

Fig. 1. Conceptual diagram of the *LepiX* model. The length of the individual larval stage is simulated by daily temperature values (degree-days), and the exposure to *Bt* pollen is related to the deposition of pollen on leaves. Both processes determine the total intake of pollen by an individual larva and result in an individual risk of mortality derived from the assumed dose-response curve.

2.2.2. Sub-models in detail

Weather module:

The weather module provides the mean daily temperature $T(t)$ for a day (of year) t in the run of one year. For the two example study sites Rheinfelden (Baden-Württemberg, Germany) and Bad Hersfeld (Hesse, Germany), we used series of daily temperature measurements provided by the German Weather Service (DWD) for the years 1961–2010. For each simulation run, one specific series of measurements over the period of one year was randomly chosen, i.e. for one simulation run, the 1000 individuals considered experienced the same daily temperatures. These daily temperatures $T(t)$ determine the development time of one larva by means of a degree-days approach, as described in the individual model below.

Individual module:

The individual model considers the phenology of butterfly larvae. In this module, both the day of occurrence and the duration of the larval period from hatching until pupation are determined for each larva. This module uses as input data a start date for the hatching of a larva from an egg ($t_{0,L}$, denoted by an ID code L) and the daily temperature series $T(t)$ from the weather module.

$t_{0,L}$ is drawn for each larva individually from a beta distribution, where the range of the distribution, i.e. the possible earliest and latest days for hatching, is obtained from published phenology data, based on field observations for the species considered. If multiple butterfly generations occur per year in the corresponding location, the module considers multiple hatching periods. In the following, the earliest day of hatching of a generation is denoted by $t_{H,begin}$. The duration of larval period Δt_L of larva L is calculated from the weather data and the simple degree-days approach (Baskerville and Emin, 1969), i.e. the daily, non-negative differences between temperature $T(t)$ and a given threshold temperature T_{min} are accumulated until a limit degree-days temperature $T_{DD,Larva}$ is reached. In recursive form, this reads as

$$T^{accum}(t) = T^{accum}(t-1) + \max\{T(t) - T_{min}; 0\} \quad \text{for } t > t_{0,L}$$

and $T^{accum}(t_{0,L}) = \max\{T(t_{0,L}) - T_{min}; 0\}$.

The day t', where $T^{accum}(t')$ exceeds $T_{DD,Larva}$, is assumed to set the day of (individual) pupation; thus, the larval period of an individual L has the length of $\Delta t_L = t' - t_{0,L}$.

The length of the individual pupal period Δt_P is calculated analogously to the larval period with the same temperature threshold T_{min}, but with a different limit degree-days temperature $T_{DD,Pupa}$.

In summary, this module defines the following state variables for each butterfly larva: time of hatching $t_{0,L}$, duration of larval period Δt_L, and duration of its pupal period Δt_P. The end of the larval instar is $t_{end,L} = t_{0,L} + \Delta t_L$, and the emergence of imagines is at $t_{end,P} = t_{0,L} + \Delta t_L + \Delta t_P$.

Deposition module:

Maize pollen deposition was derived from a large dataset (Hofmann et al., 2014) obtained by standardized technical sampling (PMF mass filter; standardized via VDI 4330-3 and CEN-TS 16817-1) covering 10 years and different locations, mostly in Germany but also including sites in Belgium and Switzerland. Each of the 214 sampling points used in Hofmann et al. (2014) represents the integrated pollen deposition for one sampling point over the entire flowering period and thus approximates the total amount of pollen deposited at this location. All these data reflect the common maize cultivation practices in Germany, which also should be expected if *Bt* maize is commercialized. The deposition module simulates the maize pollen flow during the flowering period and the deposition of maize pollen on host plants for each day t and for different distances d from a maize field. The host plant used in our case was nettle (*Urtica dioica* L.). The amount of pollen was transformed to the amount of pollen accumulated on host plants (i.e. nettles leaves) by calibrating PMF data to field data of pollen deposition on host plants (Hofmann et al., 2016).

Maize pollen deposition dependent on the distance to the maize field:

According to Hofmann et al. (2014), the mean amount μ of maize pollen deposition, as measured by PMF samplers, follows a power law function dependent on the distance d from the nearest maize field:

$$\mu(d) = 127 \cdot d^{-0.585}$$

(note that dimensions are neglected). This standardized integrated deposition measured by a PMF mass filter quantifies the total amount of maize pollen over the entire maize flowering period. With the usual cultivation practices in Germany, the standardized integrated deposition varies according to regional and local differences (e.g. climate, soil, sowing date, seed varieties). Taking this variation into account, the integrated pollen deposition variation at a given distance d follows a log-normal distribution

$$p(d) \sim LN(\mu(d), \sigma(d)),$$

where $\mu(d)$, and $\sigma(d)$ can be derived from the mean and the confidence intervals of the datasets, respectively, provided by Hofmann et al. (2014). According to the distance d, the value of $p(d)$ is chosen randomly for each simulation run.

Temporal distribution of pollen deposition:

Pollen release during the flowering period is not constant but depends on climatic conditions. A diurnal pattern is typical for the release of pollen and is correlated to turbulent weather conditions during the day (Boehm et al., 2008; Hofmann et al., 2013). Unfavourable weather conditions regularly cause interruptions of the period in which maize pollen is released, which leads to a prolonged period of pollen dispersal rather than a compact period (Hofmann et al., 2013). Long-term measurements of maize pollen are available from Ganderkesee (Lower Saxony, Germany) for the years 1994 to 2010 (data kindly provided by R. Wachter, Pollenflug Nord, in cooperation with F. Hofmann; see also Hofmann et al. (2008, 2013)). These temporally resolved periods of maize pollen deposition were used to simulate the variability of the temporal pattern of maize pollen over the flowering period.

In the simulation, how the actual total pollen amount at a distance d is distributed over the days in a year comprises two aspects. First, the starting day of pollen shedding has to be set. Second, one has to determine for each day which proportion of the actual total amount of pollen is disposed per day, i.e. the temporal pattern of pollen distribution in a simulation year. As a basis for our model approach, we used long-term data of the temporal maize pollen shedding for the site Ganderkesee (see above). These data encompass the time span over which the pollen concentration in the air $H_{1994}(t), ..., H_{2010}(t)$ was measured per day t in 1 of 17 consecutive years (1994–2010).

The starting date t_S of the temporal flowering pattern for a simulation year is drawn from a uniform distribution

$$t_S \in U(t_{S,begin}, t_{S,end}),$$

where $t_{S,begin}$ and $t_{S,end}$ is the earliest and latest day in a year, respectively, on which pollen was observed in the field.

In order to simulate realistic temporal patterns of pollen deposition, the relative proportion of pollen that is deposited per day is calculated from each of the 17 datasets of Hofmann et al. (2009). Thus, we obtained 17 different temporal patterns $h_{1994}(t), ..., h_{2010}(t)$ for the <u>relative</u> daily pollen deposition, each of which fulfill

$$\sum_{t=0}^{364} h_n(t) = 1,$$

where n denotes the year of measurement 1994, ..., 2010.

For each year in a simulation run, a specific temporal pattern $h_n(t)$ is randomly chosen. But as described above, the starting day t_S for shedding is randomly distributed. Therefore, we have to shift the values of $h_n(t)$ in time such that the beginning of shedding in the chosen temporal pattern $h_n(t)$ coincides with the chosen starting day t_S. We denote these shifted temporal patterns as $h_{n,t_S}(t)$. In this way, we have

at distance d

$$P_{air,d}(t) = p(d) \cdot h_{n,t_S}(t),$$

i.e. the simulated pollen deposition at day t in a simulation year for a given starting day t_S.

Estimation of pollen deposition on leaves:

Since the daily amount of pollen in the air determines the amount of pollen on host-plant leaves, it is necessary to relate PMF estimates of pollen deposition (air) to pollen deposition on the acceptor surface (leaf). In the model, this is achieved by the factor γ, which has been determined in field experiments by using different methods in parallel (Hofmann et al., 2016). The daily amount of pollen on the leaves is assumed to accumulate on the host-plant leaves, and therefore, the deposition amounts from the previous day must be taken into account. However, we assumed that the amount of pollen of the previous day only partly remains on the host leaves because of wind and/or rain. To simulate this decline in pollen density over time, a loss rate ε is introduced, which describes the relative amount of pollen that is lost per day.

In summary, the mean deposition on day t on a host-plant leaf at distance d for a year is

$$\overline{P_{leaf}(t)} = \overline{P_{leaf}(t-1)} \cdot (1-\varepsilon) + \gamma \cdot P_{air,d}(t).$$

Pollen is not uniformly distributed on host-plant leaves (Hofmann et al., 2011, 2016). Hofmann et al. (2016) have shown that the spatial distribution of maize pollen on host-plant leaves can be described by a log-normal density distribution with specific parameters for different plant species. Because butterfly larvae, especially young and thus sensitive instars, feed only on a small fraction of leaves, the spatial distribution of pollen will ultimately influence the exposure of larvae to pollen and thus the magnitude of effects. As dose and effect are nonlinearly related (see mortality module), it is important to include the spatial variability of pollen densities on host-plant leaves (Hofmann et al., 2016).

The data of Hofmann et al. (2016) motivates the use of a log-normal distribution, which uses $\overline{P_{leaf}(t)}$ as μ parameter and a constant σ_{leaf} parameter. Thus, the pollen amount $P_{leaf,i}(t)$ *on a leaf i* is considered to be a derivative:

$$P_{leaf,i}(t) \sim LN(\overline{P_{leaf}(t)}, \sigma_{leaf}).$$

Exposure module:

The exposure module estimates the amount of pollen per cm^2 that each larva is exposed to. This information is required for determining the risk of mortality (see next section). In determining the pollen exposure for each larva in a simulation run, we made two assumptions: (i) each larva feeds only on one particular part of a leaf per day and (ii) the pollen exposure of a larva L results from the deposition values $P_{leaf,i}(t)$ of a number of m consecutive days, as described in detail in the following.

According to the first assumption, the daily deposition of pollen $P_L(t)$ on the position where a larva L is located is $P_L(t) = P_{leaf,i}(t)$, where $P_{leaf,i}(t)$ is provided by the deposition module. These values are used to calculate the exposure A_L of the specific larva using the equation

$$A_L = \frac{1}{4} \max \left(\sum_{t}^{t+4} P_L(t) \right)$$

for every $t_S \in (t_{0,L}, ..., t_{end,L} - 4)$.

A_L is actually the mean pollen deposition on leaves derived from the sum of pollen deposition during the time period m. The time period m was set to 4 days in our example as a proxy for the exposure time that elicits effects from the uptake of *Bt* pollen.

Mortality module:

The pollen exposure of individual larva A_L (as described in the previous section) is the basis for deriving the risk of mortality of an individual larva. We assume in our model that each individual larva can only be subjected once to mortality caused by the ingestion of *Bt* pollen.

In order to estimate the toxic effect of *Bt* pollen on a given species c, the slope and the LC$_{50}$ value from an empirical dose-response relationship of pollen of the relevant *Bt* maize event and the butterfly species are needed. We used a Hill equation as a dose-response relationship (Frank, 2013) to calculate the mortality M_c dependent on the amount of pollen A_L to which a larva of the species c is exposed:

$$M_c(A_L) = \frac{1}{1 + \left(\frac{A_L}{LC_{50,c}}\right)^{-slope}}.$$

This individual mortality is used to determine the mortalities of a population of 1000 larvae in a simulation run and the mean mortality of 1000 runs, i.e. 1000 simulated populations are considered as an estimator of the mean risk of mortality for one particular parameter setting (scenario).

3. Case example: calibration and settings

The *LepiX* model can, in principle, be used for any butterfly species, geographical region and *Bt* maize event. We calibrated the model for *Bt* maize event MON810, which produces the lepidopteran-active *Bt* protein Cry1Ab. MON810 maize is the only *Bt* maize currently approved for cultivation in the EU. The lepidopteran species *I. io* used in our case example (Bryant et al., 2000, 2002; Ebert and Rennwald, 1991) is common in agricultural areas of Europe and is protected in some EU member states (Holst et al., 2013b). *I. io* also has been the subject of earlier risk assessments and is used as a model species (EFSA, 2009; Holst et al., 2013b; Perry et al., 2010). Different estimates of the toxicity of MON810 pollen for *I. io* have been found (see Table 1 below).

3.1. Geographical regions

We chose two locations for our study. In Bad Hersfeld (50°52′12″N, 9°42′7″E) in central Germany, *I. io* is univoltine; in Rheinfelden (47°35′23″N, 7°46′8″E) close to the Swiss border, *I. io* is bivoltine (Ebert and Rennwald, 1991).

3.2. Individual module

Because generally few phenotypic data of larval stages are available in the literature, we calculated the beginning of the larval period based on phenotypic data of imagines as follows. We chose the earliest egg hatch date $t_{H,begin}$, such that the simulated occurrence patterns of the imagines matches the phenotypic observations given in the literature (Ebert and Rennwald, 1991). Thus, for Bad Hersfeld, the value of $t_{H,begin}$ was set to 19 May, and for Rheinfelden, the value of $t_{H,begin}$ of the first larval generation was set to 15 May and the value of the second

Table 1
Parameter values of the dose-response curve used to estimate mortality of *I. io* larvae after exposure to MON810 pollen.

Parameter	Value	Reference
LC$_{50}$ [pollen/cm^2]	5800	Felke et al. (2010) (*Bt*176 pollen); scaled to MON810 in Perry et al., 2010
	2972	Felke et al. (2010); scaled to MON810 in Holst et al. (2013b);
	1351	Lauber (2011) in Holst et al. (2013b)
	451	Precautionary value estimated in Holst et al. (2013b)
Slope (logit[a])	−1.76	Holst et al. (2013b)
	−0.84	(probit 1.095) Saeglitz et al. (2006) used in Perry et al. (2010, 2012)

[a] Matching the Hill equation.

generation was set to 20 July.

The temperature sums for larval and pupal development were 315.2 and 110.0 degree-days, respectively, with a lower temperature threshold of 8.3 °C (Bryant et al., 2002).

3.3. Deposition module

We chose the earliest day for the beginning of pollen shedding ($t_{S,begin}$) as 14 July and the latest day ($t_{S,end}$) as 5 August. These dates represent the earliest and latest dates recorded in long-term measurements of the temporal distribution of maize pollen (see deposition module in Section 2.2.2). The ratio γ of pollen flow in the air to pollen deposition on the host-plant leaves (nettle) was set to 0.68, as given in Hofmann et al. (2016).

Only few data are available for estimating the daily loss rate ε of pollen on plant leaves. To obtain a realistic value of ε, we compared distributions of on-leaf pollen deposition provided by field measurements (Hofmann et al., 2016) with simulated distributions. We identified $\varepsilon = 20\%$ pollen loss per day as a reasonable value for ε in our simulations. This value is in the range of other estimates, e.g. Holst et al. (2013b), although the standard deviation of the simulated distributions was slightly lower than that of the field data of Hofmann et al. (2016).

3.4. Mortality module

Different estimates of the dose-response curve of *I. io* to pollen containing Cry1Ab from MON810 have been determined. To take this source of uncertainty into account, we ran the model with different combinations of the various LC_{50} values and the two slopes of the dose-response curve (Table 1 and Fig. 2).

When necessary, the slope parameter *slope* was transformed from a probit-response relationship to the Hill equation (Perry et al., 2010).
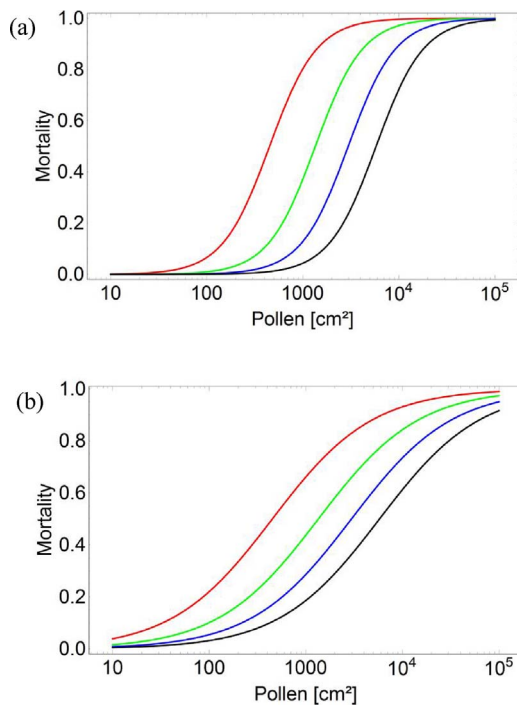
Fig. 2. Dose-response curves for different LC_{50} values (red: 451; green: 1351; blue: 2972; black: 5800 Pollen/cm²; see Table 1) with (a) *slope* $= -1.76$ (Holst et al., 2013b) and (b) *slope* $= -0.84$ (Perry et al., 2010, 2012; Saeglitz et al., 2006). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
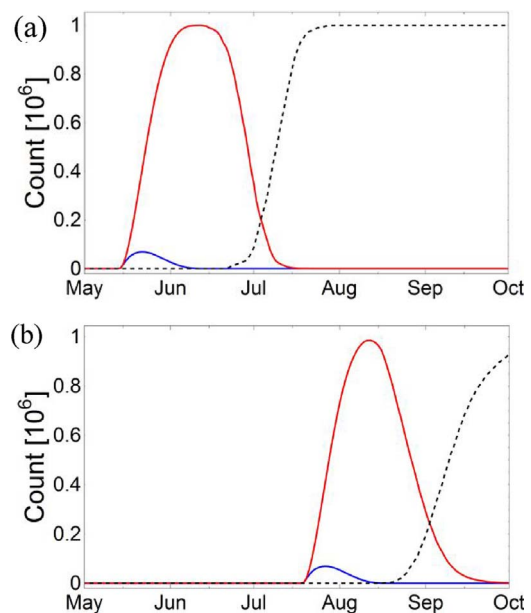
Fig. 3. Simulated phenology of *I. io* for climatic data of Rheinfelden, Germany for simulations with 1000 individuals over 1000 populations. (a) First generation; (b) second generation. Blue: number of hatching individuals; red: number of larvae; black (dashed): number of imagines. The earliest hatching date $t_{H,begin}$ of the first generation was set to 15 May and that of the second generation was set to 20 July. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Results

According to the calibration of the model for *I. io* and the two geographical regions, we performed various simulations to validate the model, estimate rates of mortality and analyse the sensitivity of the model results according to the input parameters.

The output of the individual module simulating the phenology of *I. io* was in good agreement with field data (Ebert and Rennwald, 1991) for larval and adult phenology in the regions where univoltine (e.g. Bad Hersfeld) or bivoltine (e.g. Rheinfelden) larval generations of *I. io* can be expected. This corroborates the phenology module in which a simple degree-days model and literature data (Bryant et al., 2000, 2002) have been used to simulate the phenology of *I. io*. According to Ebert and Rennwald (1991), the first larval generation of *I. io* can be observed in the upper Rhine valley from May to the beginning of July, and the second larval generation can be observed from August to September, with some larvae developing in October or November in extreme years. The imagines from the first and second larval generation can be sighted typically in July and from the end of August through October, respectively. We simulated larval and adult phenologies for both generations using the climatic data of Rheinfelden (Fig. 3). We found a good correspondence between the observed and simulated times for the occurrence of both larvae and imagines. Especially the simulated larval phenology fit the observations well and confirmed (*sensu* Oreskes et al., 1994) our approach.

To estimate the average risk of mortality of an individual of *I. io* dependent on the distance to a field of *Bt* maize, we simulated 1000 populations of 1000 individuals each (see Section 2.2.1). Simulations showed no apparent ($< 1\%$) mortality rates with the climatic conditions in Bad Hersfeld, where *I. io* is univoltine (data not shown). With climatic data from Rheinfelden, where *I. io* is bivoltine, the simulations predicted low mortality rates of the first larval generation of *I. io* similar to those of Bad Hersfeld, but higher mortality rates ($> 1\%$) for the second larval generation, depending on the distance to the maize field and toxicological input data (Fig. 4). We used a threshold of 1% mortality, as this threshold is currently used in the risk assessment of *Bt*
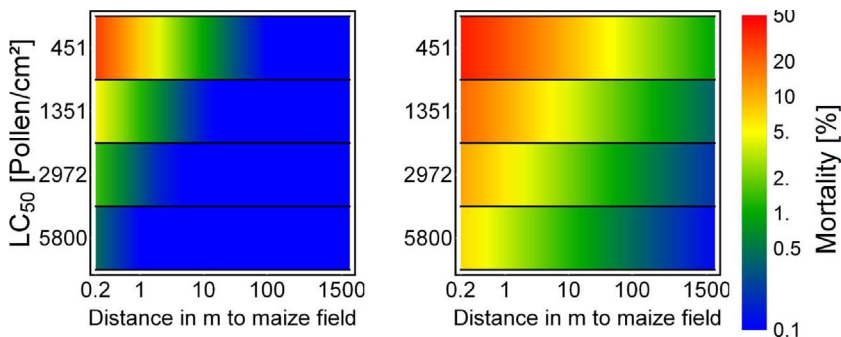
Fig. 4. Relationships of larval mortality to distance to maize field and to LC$_{50}$ values. Left: *slope* = −1.76 (Holst et al., 2013a,b); right: *slope* = −0.84 (Saeglitz et al., 2006; Perry et al., 2010, 2012). Simulated mortalities of second generation *I. io* larvae in Rheinfelden are shown at different distances from a *Bt* maize field and for different dose-response curves, i.e. different LC$_{50}$ and slope values. (For interpretation of the colors in this figure, the reader is referred to the web version of this article.)

maize at the EU level (EFSA, 2015). Both the distance and the eco-toxicological parameters influenced the risk of mortality (Fig. 4). Mortality decreased with increasing distance to the maize field and with decreasing sensitivity (increasing LC$_{50}$). Furthermore, mortality estimates depended on the slope as an input parameter to describe the dose-response equation. Steeper slopes in this respect decreased mortality estimates. Because of non-linear relationships, this decrease was more distinct with increasing distance to the maize field. For example, assuming an LC$_{50}$ value of 2972 pollen/cm^2, mortalities of 5.9% and 0.3% were predicted at a distance of 1 m, whereas mortalities of 0.75% and 0.04% were predicted at a distance of 100 m for the applied slopes of −0.84 and −1.76, respectively (see also sensitivity analysis below).

To test the robustness of the model results presented above against the chosen values for the model parameters, we performed a two-stage sensitivity analysis. We analysed simulations of the second generation of *I. io* in Rheinfelden, which had the highest mortality in our simulations. In the first step, we identified parameters that had the most severe impact on the results. For this purpose, for each of the four different LC$_{50}$ values, we used a Latin hypercube sampling with uniform stratified parameter values for nearly all model parameters (Saltelli et al., 2008) and simulated a total of 1000 simulations runs (data not shown). The analyses indicated that besides the LC$_{50}$ value, whose impact on mortality is obvious, the average individual risks of mortality are most strongly influenced by the parameters *slope* in the dose-response curve and the earliest day of hatching (parameter $t_{H,begin}$) of the second generation.

In the second step, the two parameters (*slope* and $t_{H,begin}$) were varied systematically in combination to test for their impact on mortality risks. The risk of mortality invariably decreased with steeper slopes for different dates of first hatching (Fig. 5). Complementary, the mortality rates for different slopes depicted a maximum at ca. $t_{H,begin}$ = 200 days, i.e.18 July. This date mirrors a maximum overlap of
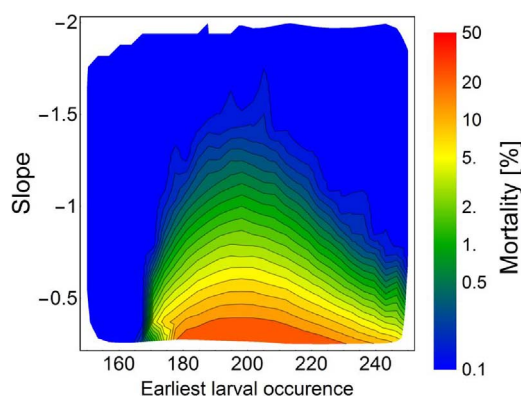
larvae occurrence and pollen shedding.

The mean mortality rates as shown in Figs. 4 and 5 were derived for each parameter set from a sample of 1000 simulated populations, each providing one mean individual mortality rate within the population. The differences between the simulated populations are solely due to different realizations of random processes, i.e. weather and individual hatching times (see section 2.2.2). To estimate the variation in individual mortality rates, we looked for the variation in risks of mortality within single populations. As an example, Fig. 6a demonstrates for a specific parameter setting with a distance of 40 m from the *Bt* maize field the distribution of 1000 mean individual mortality values gained from 1000 simulated populations. In this example, the mean mortality risk was about 25%, but it varied from 0 to about 50%. When we considered the total sample of 1000 × 1000 pooled individuals from all simulated populations for one specific distance to the field, we observed a multimodal distribution with a distinct maximum in the class of nearly zero mortality (Fig. 6b), where approximately 10% of all individuals do not notably suffer mortality. The remaining individuals were assigned to risks of mortality ranging from 1 to approximately 80%.

The graphs in Fig. 6c and d exemplify the distribution of individual mortalities for two particular populations using the identical input parameters as in Fig. 6b. Although both populations belong to the same sample that leads to an *average* individual mortality risk of about 25%, the simulations demonstrate that different distributions can be observed: In one run, the majority of individuals of a population hardly suffered any mortality (Fig. 6c), whereas in other runs, individual mortality covered the entire range between 0 and 1 (Fig. 6d). Note, that this striking difference is solely due to random effects.

5. Discussion

5.1. General remarks

The *LepiX* model presented here is an individual-based model designed to estimate the risk of mortality of non-target butterfly larvae caused by exposure to *Bt* toxins expressed in pollen of insect-resistant *Bt* maize. *LepiX* can be easily applied to other Bt maize events (both single and stacked events) and to other species of Lepidoptera. The specific data needed should, in principle, be available from the scientific literature and from market applications of the respective *Bt* maize. The conceptual model can be also applied for crop species other than maize and different taxa of non-target organisms.

The agent-based approach in the *LepiX* model allows explicit consideration of the impact of variability on the estimation of the individual risk of mortality. In the *LepiX* model, variability is considered in nearly all features: weather (temperature), occurrence of larvae, pollen deposition and individual exposure. This variability in the processes finds expression in stochastic distributions for the output variables and provides a much more distinct interpretation of model outcomes than the use of just mean values alone. In this way, *LepiX* is suitable for assessing individual mortality probabilities, which might

Fig. 5. Sensitivity analysis for a given LC$_{50}$ value of 1351 pollen/cm^2. The values of the slope in the dose-response curve (cf. Table 1) and of the earliest day of larval hatching (parameter $t_{H,begin}$) for the second larval generation of *I. io* in Rheinfelden are systematically varied. (For interpretation of the colors in this figure, the reader is referred to the web version of this article.)
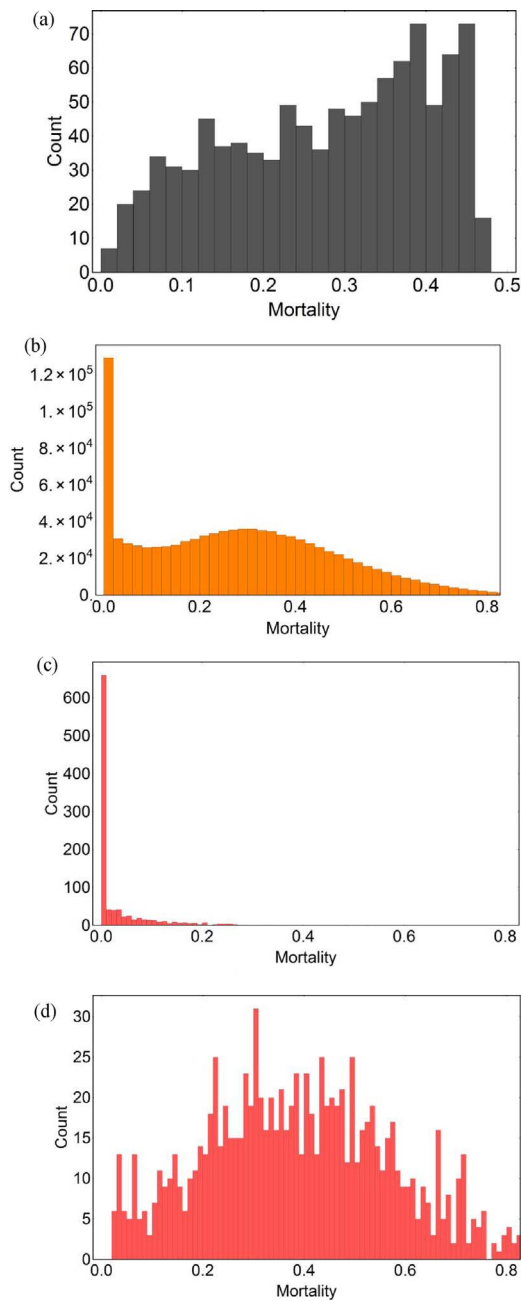
Fig. 6. Simulation results for a *slope* of −0.84, a distance to the maize field of 0.2 m and an LC_{50} of 451 pollen/cm^2 (mean mortality value: ca. 25%; see Fig. 4). (a) Histogram of the mean mortality values from 1000 simulated populations; (b) histogram of the total of 1000 × 1000 simulated individuals; (c) and (d) examples of individual mortalities for two particular simulated populations (both simulated for identical input parameter settings).

need to be considered for strictly protected species. The ability to assess such individual mortality probabilities is one major advantage of the use of agent-based models (Grimm and Railsback, 2006). In this respect, *LepiX* differs from the model currently used in the EU risk assessment (EFSA, 2015; Perry et al., 2010, 2012).

LepiX provides not only quantitative values for important output variables (such as the risk of mortality), but also constitutes a valuable tool for investigation of the sensitivity of model assumptions to these variables. The model can be used to directly compare different scenarios and parameter sets in view of the output variables (see below).

5.2. Comparison to other approaches

To date, two other models for estimating lepidopteran larvae mortality caused by *Bt* maize pollen have been published (see Introduction): the EFSA model (EFSA, 2015; Perry et al., 2010, 2012) and the *BtButTox* model (Holst et al., 2013a,b). Like the EFSA model, *LepiX* can be used to take various distances to the *Bt* maize field into account. All three models use a dose-response curve to calculate mortality and do not consider sub-lethal effects. In contrast to the EFSA model (Perry et al., 2012), *LepiX* does not make any assumptions on the distribution of host plants, butterfly populations or adoption of *Bt* maize cultivation. Any comparisons of *LepiX* to the EFSA model must therefore be made to the present EFSA *local model* (EFSA, 2015).

Only the *BtButTox* and *LepiX* models simulate the temporal coincidence of larvae and maize pollen. To do so, an insect module and a deposition module are required. Holst et al. (2013b) used a full-scale insect model requiring detailed biological input, whereas we reduced biological input data used in *LepiX* to reflect the lack of availability of detailed data on, e.g. the lifespan or the pre-oviposition period, for most butterfly species. Instead, the phenology module of *LepiX* is mainly driven by a simple degree-days model and relies on field observation data on the adult flight period, which is available for many species. A single observation of the flight activity of a species can also be used to derive accumulated degree-days sums for a species if weather data for the region can be obtained. Although larval development is variable and in part dependent on environmental and genetic factors (Friberg et al., 2011; Saastamoinen et al., 2013), degree-days calculation are useful for predicting phenology of butterflies across species (Cayton et al., 2015).

The *BtButTox* model does not include a distance relationship of the deposition of maize pollen and the maize field. By contrast, both the current EFSA model (EFSA, 2015) and the *LepiX* model use, in principle, the same distance relationship of Hofmann et al. (2014).

Compared to other approaches, *LepiX* uses the so far most comprehensive set of field data to estimate pollen deposition on plant leaves and is the only model that accounts for the variability of pollen on leaves. Compared to Holst et al. (2013b), who approximated pollen deposition on leaves using Durham samplers that operated over one single year and in one single location in Japan (Kawashima et al., 2004), both the *LepiX* and EFSA models derive pollen deposition on leaves from long-term monitoring data obtained using standardized technical samplers (Hofmann et al., 2014). To translate pollen counts from technical samplers to acceptor specific pollen deposition on leaves of specific host plants, we followed the approach of Hofmann et al. (2016) who experimentally compared the efficacy of different sampling methods and thus was able to calibrate PMF measurements to in-situ field measurements of pollen deposition on host plant leaves. In contrast to the EFSA model (EFSA, 2015), this has the advantage of replacing expert estimates with field data. In addition, we included the variability of pollen deposition among leaves. As dose-response effects are basically non-linear, it is important to consider this variability explicitly for a reliable risk assessment.

The results of modelling larval mortality using the *LepiX* and *BtButTox* models can only be partially compared because the conceptual models and the model formulations differ. The results of the two models for *I. io* are in good agreement in that they predict no apparent mortality for the first generation of *I. io* regardless of the chosen climatic data. Similar to the *BtButTox* results of Holst et al. (2013b), the lack of mortality effects predicted by *LepiX* can be explained by the lack of overlap between pollen shedding and larval occurrence. For the second larval generation in Rheinfelden, however, the importance of this overlap is reflected in the sensitivity analysis of model parameters, which stresses the importance of the beginning of the larval period for the prediction of mortality. With identical dose-response parameters (slope = −1.76; LC_{50} = 451 pollen/cm^2), the *LepiX* model predicts 28% mortality for *I. io* larvae feeding on host plants at the field edge

(0.2 m). This value is on the lower end but within the range of the estimates (28–86%) of Holst et al. (2013a,b), taking into account different assumptions for pollen deposition rates of the *BtButTox* model.

With parameter settings for the dose-response similar to those of the EFSA model (slope = −0.84; LC_{50} = 5800 pollen/cm^2), the *LepiX* model predicts 6.9% mortality for *I. io* larvae feeding on host plants at the field edge (0.2 m). Although a direct comparison of estimates with the current version of the EFSA model (EFSA 2015) is difficult, we can compare the estimates indirectly by using suggested mitigation measures to keep mortalities of larvae < 1%. To achieve this threshold, EFSA (2015) recommends isolation distances of 5 m to 20 m, depending on the parameter setting. Our results for *I. io* are in keeping with this; *LepiX* predicts a mortality of *I. io* of 0.9% at a distance of 20 m and a moderately higher mortality of 1.7% at a distance of 5 m from the maize field. It is important to note, however, that some butterfly species have a higher sensitivity to MON810 pollen (Cry1Ab toxin). For native species in Europe this has been demonstrated, e.g. for the protected Common Swallowtail (*Papilio machaon* L.) (Lang and Vojtech, 2006) or for the diamondback moth *Plutella xystostella*. Especially *P. xystostella* has been reported to be highly sensitive to Cry1 and thus will better perform as a surrogate for highly sensitive species (Felke et al., 2002; Wolt et al., 2005). Using the EFSA correction factor of 31.05 (Perry et al., 2010) to adjust the toxin concentration from Bt176 pollen to MON810 pollen, the LC_{50} value of *P xystotella* larvae (L4) is estimated to be 595 pollen/cm^2 (Felke et al., 2002), and larger isolation distances will apply for the risk management (see Table 2).

5.3. Implications of uncertainty

Although the output of *LepiX* is in agreement with previous calculations, the modelling results also highlight the importance of input data for the model outcome. To date, the scientific discussion in this context has mainly focused on exposure estimates (EFSA, 2015, 2016b; Hofmann et al., 2014, 2016; Kruse-Plass et al., 2017; Perry et al., 2013), and it is agreed that the exposure estimate will have a strong influence on any model predicting the effects of the exposure of non-target organisms to *Bt* pollen. However, our simulations demonstrate that both LC_{50} and the slope of the dose-response curve strongly influence mortalities and thus envisaged risk management measures. These findings agree with those of Perry et al. (2012), who stressed the importance of these parameters for the EFSA model. As has been shown in Figs. 4 and 5 both the slope and the LC_{50} value of the dose response as well as the timing of larval development will influence the mortality estimate. Imprecisions in these model parameter, in consequence, will affect not only the mortality estimate but also any risk management measure using this estimate.

In our case example, a less-steep slope (−0.84) of the dose-response curve caused higher mortality of *I. io* larvae, regardless of the assumed LC_{50} value or the distance to the maize field. Compared to a steep slope, a less-steep slope will increase the mortality estimate if the environmental concentration of the stressor (*Bt* protein in pollen) is lower than the LC_{50} value. As the distance relationship of maize pollen follows a power function, larger distances will increase the probability that exposure levels are below the LC_{50}, even for highly sensitive species. In our case example, this can be illustrated by comparing the mortality estimates for different slopes at a given distance. Assuming an LC_{50} value of 1351 pollen/cm^2, the less-steep slope (−0.84), in comparison to the steeper slope (−1.76), results in about a 4-, 30-, 97-, and 220-fold increase in mortality for distances of 0.2, 10, 100 and 500 m, respectively. Our modelling results affirm the use of less-steep slope estimates for the risk assessment in order not to underestimate effects (Perry et al., 2010). This may also be reasonable as the influence of sublethal effects, such as reduced weight (and presumably fitness) or prolonged developmental time on individual fitness or population growth, have not yet been included in simulation models.

To date no standardized ecotoxicity testing for Lepidoptera exists which takes exposure of larvae to toxic pollen into account. The lack of agreed methodology causes considerable uncertainties which have been addressed in detail in Lang and Otto (2010). In fact, in experiments carried out to determine LC_{50} values for *Bt* toxins and Lepidoptera, some of the used experimental parameters, such as exposure time, the time of mortality readings or the lack of secondary stressors, have been criticized to underestimate real effects (Lang et al., 2011). As *LepiX* can rely only on available data this uncertainty has to be addressed in the final risk assessment and risk management.

Provided that suitable data exist, some uncertainties in LC_{50} values can be addressed by the use of species sensitivity distributions (Posthuma et al., 2002). This approach has been followed in the current EFSA model (EFSA, 2015; Perry et al., 2012) and is useful for estimating, e.g. effects on species of conservation concern for which ecotoxicological data are lacking.

Both LC_{50} and slope estimates, and to a minor extent estimates of larval phenology, will influence risk management measures. We illustrate this in Table 2, which provides isolation distances, based on *LepiX* calculations, needed to obtain mortality estimates for *I. io* larvae < 1% (see above). When the steep slope is used, changes in the LC_{50} value do not alter isolation distances greatly, i.e. all distances lie within one order of magnitude. When the gentler slope in *LepiX* is used, however, isolation distances greatly increase to roughly two orders of magnitude. A less-steep slope and the uncertainties in LC_{50} values resulted in estimated isolation distances range from 20 m to 2000 m.

5.4. Implications of individual and population variability

The individual-based *LepiX* model provides especially insight into the variation in mortality to be expected for a single larva or single populations. Such analyses are useful when impacts on protected butterfly species need to be taken into account. Many protected species have a limited potential for recovery and are already under threat by multiple stressors (EFSA, 2016a). In addition, many of these species are rare and only occur in small populations and thus are highly vulnerable to extinction. For this reason, the relevant protection goal may need to focus on single populations, or in the case of highly protected species, on single individuals of a population. The variability of the mortality

Table 2

Consequences of uncertainties in biological data for risk management measures (isolation distances) to minimize risks *for Inachis io* 2nd generation larvae (protection level 1% mortality) from *Bt* maize (MON810) cultivation: Influence of dose-response parameters and hatching time. Values: isolation distances [m] from the nearest maize field.

LC$_{50}$ value [pollen/cm^2]	Dose-response slope = −1.76					Dose-response slope = −0.84				
	Start 2nd larval generation					Start 2nd larval generation				
	1 July	10 July	20 July	1 Aug	15 Aug	1 July	10 July	20 July	1 Aug	15 Aug
5800	0.2	0.2	0.2	0.2	0.2	5	15	20	10	2
2972	0.2	0.5	0.5	0.2	0.2	20	50	60	30	5
1351	1	2	2	1	0.5	70	180	300	120	15
451	5	10	10	10	5	200	1500	2000	900	120

rates on the population level varied from 0 to 50% (Fig. 6a), the variability on the individual level was much higher (Fig. 6b). Although the overall *average* mortality rate determined in our case example was about 25%, random effects alone can lead to quite different outcomes for the same specific input parameter set in different model populations as demonstrated in Fig. 6c and d. Therefore, it may be misleading to consider only average mortality rates, especially in the context of conservation of single individuals or populations.

6. Conclusions

We present *LepiX* as a versatile tool to assist the risk assessment and risk management of *Bt* maize effects on non-target Lepidoptera. The model balances the need for detailed biological data while offering a high degree of stochasticity to account for the natural variabilities for all three model components: i) pollen deposition and exposure, ii) larval phenology and iii) larval mortality. *LepiX* allows modelling the effect of larval phenology on the likelihood of exposure to maize pollen and can be used to derive risk management measures in terms of isolation distances. However, the definition of such distances will depend on the amount of uncertainties that regulators and risk managers are willing to accept. A sensitivity analysis of our model implementation for *I. io* revealed a prominent influence of both LC_{50} value and the slope of the dose-response equation on the mortality estimate. This stresses the importance of obtaining reliable, more standardized, toxicity data before any decision can be made on the risk assessment and risk management prior to market release of *Bt* maize (Lang and Otto, 2010). Although this has been realized by the EFSA GMO panel (Perry et al., 2012) as the EU regulatory authority, very few data, especially on non-pest species, are available in technical dossiers or in public literature. Our results strongly speak in favour of reliable ecotoxicological data that *"… should be routinely calculated and reported, together with estimates of their variability"* (Perry et al., 2012). Without reliable data, uncertainty in risk management measures can be high and respective measures can be difficult to defend.

The individual-based approach provides insights in the variability of mortality estimates of individuals or single populations and stresses that mortality in single populations or of individuals can be substantially higher than the average model predictions. This aspect has so far not been fully taken into account in the assessment of risks for highly protected butterfly species and may stimulate the scientific discussion on alternative approaches to the current risk assessment practice of protected species (EFSA, 2015). *LepiX* will be further developed and is not restricted in its applications to GMOs. The model design might also be useful in a wider context for different crop plants, toxicants and non-target species. In principal, the conceptual model and its modules are suited for simulating any effect of a toxic component in pollen, such as pesticide residuals from systemic insecticides.

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