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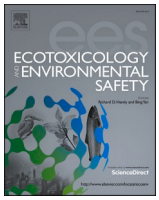

A Novel Lepidoptera bioassay analysed using a reduced GUTS model

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ABSTRACT

Lepidopteran species can be both pests and also beneficial pollinators for agricultural crops. However, despite these important roles, the effects of pesticides on this diverse taxa are relatively understudied. To facilitate the assessment of pesticides and other chemical hazards on this taxa, we present a novel bioassay capable of testing chemical sensitivity to lepidopteran larvae through dietary exposure. We used *Mamestra brassicae* caterpillars as a model lepidopteran and tested their sensitivity for the organophosphate insecticide chlorpyrifos. We exposed larvae to an artificial diet spiked with chlorpyrifos and monitored survival over time, as well as weight change over a 96-hour exposure period. To test the repeatability and reliability of the developed bioassay, the experiment was repeated three times. The survival in time data collected enabled analysis with the General Unified Threshold of Survival (GUTS) model, recently recognized by EFSA as a ready-to-use tool for regulatory purposes. The GUTS modelling was used to derive a set of relevant toxicokinetic and toxicodynamic parameters relating to the larval response to exposure over time. We found that across the three repeats studies there was no more than a threefold difference in LC_{50} values (13.1, 18.7 and 8.1 mg/Kg) at 48 h and fourfold difference at 96 h, highlighting the repeatability of the bioassay. We also highlighted the potential of the method to observe sub-lethal effects such as changes in weight. Finally, we discuss the applications of this new bioassay method to chemical risk assessments and its potential for use in other scenarios, such as mixture or pulsed exposure testing.

1. Introduction

In agricultural landscapes, prophylactic chemical application is widely used as a means of controlling insect pest populations. However, this widespread pesticide use can also damage both the structure and function of ecosystems (Diaz et al., 2019). Pimentel (1995) estimated that less than 0.1% of applied pesticides reach their target pest while the remaining 99.9% entered into the wider environment, where they may interact with non-target organisms and beneficial species. Important non-target species, such as pollinators, can be adversely affected by pesticide exposures. Hence, it is important to understand how pesticides interact with different species from the range of different pollinator groups.

Worldwide, pollination is responsible for contributing an estimated $577 billion to the global economy (IPBES, 2019). However, due to a range of factors including chemical control practices, many pollinator populations are in decline (Goulson et al., 2015; Kessler et al., 2015). In recent years, the loss of pollinators has provoked an increase in research into the effects of agrochemicals on these taxa (Iverson et al., 2019). These studies have largely centred on honeybees. This is due to their noted value in pollination and honey production and also because their eusocial nature makes it possible to easily rear large numbers of individuals for use in testing. However, as well as bee species lepidopterans (moths and butterflies) are also important pollinators (Hahn and Bruhl, 2016). This group is, however, currently largely overlooked in studies that consider the effects of pesticide exposures on insects.

Moths are the most speciose order of lepidopteran flower visitors, providing pollination for almost 300 known plant species (MacGregor et al., 2015; Rader et al., 2020). Pollination by lepidopteran species is essential for some crops, as well as also for wildflower species. A case study demonstrating of the value of lepidopterans as agricultural pollinators showed that butterflies or moths are responsible for approximately half of all floral visits of Macadamia plants cultivated in Brazil (Santos et al., 2020). Therefore, understanding the effect of chemicals on

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lepidopterans is critical from the perspective of conserving this widely admired and important group of insects.

In addition to their role as pollinators in their adult stage, lepidopteran in their larval stages are serious pests (Reed and Pawar, 1982; Cartea et al., 2010). The Cabbage Moth (*Mamestra brassicae)* is a polyphagous species that favour *Brassicaceae* and *Chenopodiaceae* (Popov and Popova, 1993), that are found in almost all Palearctic regions (Masaki, 1968). Cabbage moths are important pests of *Brassica* crops throughout Europe and Asia (Finch and Thompson, 1992), and as such significant resources are devoted to their control. This juxtaposition between adult and larval ecology gives many lepidopterans the unusual role of being both beneficial pollinator and also pest species. Interest in understanding the effects of agrochemical exposure on lepidopterans, therefore, exists from both the perspective of controlling pests and wishing to preserve a group providing a valuable biological service. Robust and repeatable approaches for lepidopteran toxicity testing are, therefore, required to investigate sensitivity to pesticides in this group.

In many previous Lepidoptera toxicity tests, the leaf dip method has been utilised as the basis for exposure (Morse et al., 1986; Santos et al., 2011; Hill and Foster, 2000). This protocol involves dipping a section of leaf into a chemical that is then fed to the larval stage. Survival is assessed usually at a single 48-hour time point. The leaf dip method has become widely adopted for lepidopteran toxicity testing, primarily because of its ease of use. However, this method is restricted in its application as a bioassay by factors such as leaf freshness. These effects mean that this approach may not be practical for longer term monitoring. Further, the current leaf dip methods generate data only suitable for ad hoc statistics at a single time point to calculate a no observed effect concentration (NOEC), lowest observed effect concentration (LOEC), or lethal concentrations (LC_x). Such parameters cannot be extrapolated to other (shorter or longer) exposure times than that at which a specific measurement is made. As such, the results are operationally defined by the exposure length and so lack biological meaning for other time points.

To move toward a more mechanistically based approach for interpreting toxicity test results, the use of toxicokinetic-toxicodynamic (TK-TD) models was proposed more than 20 years ago (Bedaux and Kooijman, 1994). Among these TK-TD models, the General Unified Threshold model for Survival (GUTS) to predict the effect of exposure on survival over time provides an established framework for TKTD based modelling (Jager et al., 2011). Once calibrated, the GUTS model can also be used to calculate the LC_x and EC_x values of a chemical, at any time point, and predict the survival of new exposure scenarios, in both constant and pulsed exposures (Baudrot et al., 2018). Bioassay exposure systems that support GUTS modelling, thus, have far greater values for the mechanistic interpretation of chemical effects than single time point assays.

To date, there is little published work on methods to generate data suitable for TKTD analyses in insect species beyond bees, and none for lepidopterans. Here, we aimed to develop a new and enhanced technique capable of monitoring acute and sub-lethal effects of agrochemicals on *Mamestra brassicae* lepidopteran larvae by effect measurement over an increased exposure time. The bioassay was designed to be i) efficient to allow high through-put of organisms to allow high replication testing, ii) be repeatable and iii) be capable of assessing acute and sub-lethal effects over 96 h allowing a TKTD modelling approach, more specifically the use of the GUTS model, and iv) be suitable for measuring effects of sub-lethal endpoints, such as body weight change.

2. Methods

2.1. Culture of test species

The *Mamestra brassicae* larvae used were collected from a laboratory strain kept in culture for over 40 years at the UK Centre for Ecology and Hydrology. Over this time, the larvae have been raised on a modification of Hoffman's tobacco hornworm diet (Smith, 1966) - from this point referred to as 'artificial diet' (See Supp. Material for recipe). Larvae were kept in culture in a controlled room at 20°c ± 2°c under a 16:8 light: dark pattern.

During culture and in rearing for experiments, the adult populations were maintained in flight cages with continuous access to 10: 90% honey: water solution. Laid eggs were collected three times a week to ensure that the batches used for rearing were all laid within the previous 24-hour period. Eggs were then added to rearing boxes containing the artificial diet and allowed to hatch. The larvae were kept until they were three weeks old, at which time they were used either for the bioassays or raised to adults from which the next generation were breed. Larvae selected for use in a bioassay were always taken from one of the cohort of collected eggs laid on the same day.

Before any chemical testing took place, an observation of larvae growth was conducted to determine the optimum size of larvae to use for further testing. Individual plastic pots containing 10 ml artificial diet added as liquid and allowed to set. An individual 7-day old 2nd instar larva (n = 15) was placed on the surface of the diet. Larvae were weighed every 2 days and the each moult time recorded. This pilot study was multi-functional in that it highlighted the instars with greatest time between moults and also allow an assessment of weight changes without any chemical effect. As random mortality increases with time, the ideal instar for bioassays would take a short time to reach, but be large enough to notice sub-lethal weight changes and preferably have a time of at least of 96 h between moults to avoid moult taking place during the exposure. Results of this initial observation established the suitability of 4th instar larvae (Fig. 1). Larvae of this stage could be collected at 12 days post hatching and were unlikely to moult during the 96-hour test duration.

2.2. Preparation of Test Chemicals

The bioassay was designed to assess the toxicity of the tested chemical on larval survival over time. For an initial assessment of suitability and repeatability for time series survival and sub-lethal effect measurements, we chose to work with a chemical that is known to exert expected toxicity on lepidopteran species - specifically, we selected the organophosphorus insecticide chlorpyrifos. High purity analytical standard chlorpyrifos > 99% (Sigma Aldrich, Poole, UK) was used to make an initial stock diluted in acetone as a 1% solution. This master stock was then diluted in further acetone to generate the required series of exposure concentrations needed for testing. Three tests were conducted, each using differing concentration ranges. For the first bioassay, the concentrations of chlorpyrifos in media assessed were 0, 0.27, 0.82, 2.4, 7.4, 22.2, 66.6 and 200 mg/kg wet weight of artificial diet. This first test used a broad range of concentrations within the solubility range of

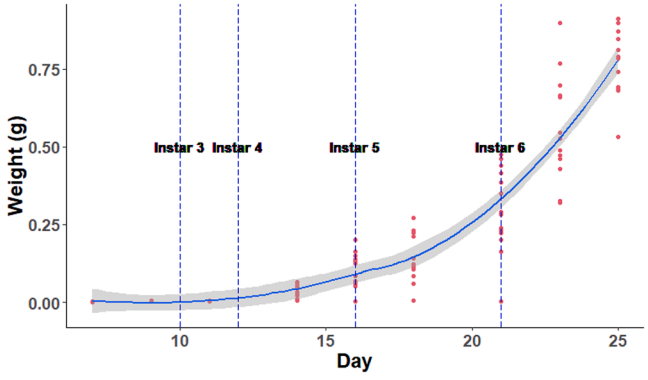

Fig. 1. Growth pattern of *Mamestra brassicae*, fed ad libitum with an artificial diet, from instar 2 to pupation (N = 15). Blue dashed lines represent the different moults. The points are the data and the line is the rolling average with 95% (confidence interval as grey shadow area).

the chemical and acted as an initial assessment of the observed toxic effect. The second test was conducted using a narrower range of concentrations of 0, 3.93, 6.29, 10.07, 16.11, 25.78, 41.25, 66 mg/kg wet weight diet, to allow a more refined assessment of the survival response over time. This range was then further refined for the final bioassay, in which concentrations of 0, 10.35, 13.46, 17.5, 22.75, 29.58, 38.46, and 50 mg/kg wet weight diet were used. In all three cases, the test medium was prepared using a new chlorpyrifos master stock prepared from the pure compound dissolved acetone. This meant that all tests were fully independent, and so their comparison can be used as a verification of the repeatability of the method and the consistency of dosing. The same volume of acetone without added chlorpyrifos was added to the control treatments and to all treatment below the maximum concentration, to ensure that any effects seen were not related to the acetone in the test medium.

2.3. Bioassay procedure

Artificial diet was made up following the same adapted recipe of Hoffman's tobacco hornworm recipe as used in culturing, but with 4000 ml of water used instead of 3200 ml. This change was made to ensure that the consistency of the diet was suitable for even dispensing to allow accurate dosing. The required amount of diet needed for all replicates for each concentration were initially weighed. The required aliquots of chlorpyrifos stock was then added to give the desired concentration in the dietary medium. After preparation, the artificial diet was kept in a water bath at a constant temperature of 50°C, to prevent setting. This short period (<2 h for each treatment) also allows a proportion of the added acetone to volatilise from the dosed diet (which can be checked by smell for the removal of solvent odour). Although the chemical, in this case chlorpyrifos, is only maintained at 50°C for a short period of time, care should be taken to ensure that the pesticide does not degrade at this temperature. Chlorpyrifos is known to be stable at temperatures up to 74°C for 3 days (Mansour et al., 2018). Hence in this case, exposure is not affected by maintenance at this temperature for the short period used. For alternative pesticides, a check on stability would be needed, although it can be expected that few active ingredient and formulation would be affected by this treatment given their designed stability for on-farm storage. All diet preparations were mixed thoroughly to ensure an even distribution of the insecticide. Then 1 ml, sufficient for ad libitum feeding, was added to each well of a 12 well plate. In total there were 24 wells per treatment. Each plate was left to cool and set at room temperature for 15 min before a single larvae was added.

All tests were conducted using 4th instar larva from the same clutch of eggs (i.e., laid on the same day), weighing (mean ± range) 20 mg ± 10 mg. This range of weights is typical for this instar in *M. brassicae*. Use of these randomly selected individuals represents the true range of individual size in the exposure better than would any selection for individuals of a specific size range. Individual larvae were placed onto the artificial diet in each well to initiate the exposure. This design gave a total of 24 individuals per treatment for survival monitoring. Mortality was measured at four time points over the 96 h of the exposure (6, 24, 72, and 96 h) by stimulating the 2nd segment behind the head of the larvae with a fine paintbrush. If no movement was detected after 5 s, the larva was recorded as dead. All plates were kept incubated at a constant 20°C for the duration of the test. This design gave a measurement of larval survival over time at each exposure concentration for use as input for TKTD based GUTS-RED-SD modelling.

2.4. Data analysis

The survival data obtained from each experiment was analysed with the openGUTS software (http://openguts.info/). We used the GUTS reduced (RED) model which combines TK and TD damage dynamics to link external concentrations to the observed effect on survival over time. The GUTS-RED model is a relatively simple derivation of the classical GUTS TK/TD modelling framework. GUTS-RED includes relatively few parameters. As such, it is an efficient model for the analysis of survival in time datasets, because it requires a relatively limited amount of time-dependent data. For example, no measurement of body residues are needed and no other information on the toxicokinetics is required. Indeed, the model can be parametrised by the survival data in time alone. GUTS-RED models provide a number of TK/TD parameters, including the no effect concentration (NEC) - a time independent metric that differs from traditional time bound toxicity metrics such as the LC_{50} (n.b. which can also be calculated from the GUTS TK/TD parameters for any timepoint). While both of these types of toxicity metric are valuable from a regulatory perspective, the NEC is considered as more ecotoxicologically relevant, because it does not change over time, unlike the LC_{50}, the value of which will depend on "when" the response is measured (Ockleford et al., 2018).

The openGUTS software provides analysis with both the stochastic death (SD) and individual tolerance (IT) approach. Briefly, both forms consider that individuals have a threshold value in terms of chemical concentration, and once this value exceeded, they die (Jager et al., 2011). The SD approach considers that all individuals have the same internal threshold concentration for effects survival (given by the no effect concentration, NEC) and that the death process is based on a statistical concept of hazard modelling, which considers that every individual has a certain chance to die (stochastic death) that increases with greater exposure to the chemical. The IT approach is based on the concept that the individuals that die at a certain time point are more sensitive (have a lower internal effect concentration threshold) than those that survive (which have a higher internal effect threshold). Within the IT model, it is, thus, considered that individuals differ in their threshold and die instantaneously once they reach it. More explanation on the comparative differences between the SD and IT approaches and their application can be found in the GUTS e-book (Jager and Ashauer, 2018). Overall, both approaches have been successfully used to provide good fits and predictions of survival data over time. In this study, we present the results of the GUTS-RED-SD model. We made this pragmatic choice because, i) overall the GUTS-RED-SD model consistently gave a slightly better fit to the bioassay data based on the model efficiency values provided by openGUTS (Table S2), and ii) the individuals used were bred in laboratory conditions for more than 40 years and are genetically similar and therefore, do not fit well with the hypothesis of the IT approach of a difference in individual internal thresholds.

In the GUTS-RED-SD model, the TK and TD are combined into a one compartment model with a dominant rate constant k_d which describes the dynamics of the "scaled" damage and will represent the one-compartment approximation of the "true" two-compartment behavior (TK and damage dynamics). The scaled damage is calculated as follows:

$$\frac{dD_w}{dt} = k_d(C_w - D_w)$$

Where D_w is the scaled damage [mg kg^{-1}], C_w [mg kg^{-1}] is the external concentration, and k_d is the dominant rate constant [d^{-1}]. Then, the death mechanism SD is calculated as follow:

$$h_z = b_w \max(0, D_w - m_w) + h_b$$

$$S = \exp(-h_z t)$$

Where h_z is the hazard rate [1/d], b_w is the killing rate [1/mg kg^{-1} d^{-1}], m_w (i.e., the NEC) is the damage threshold for effects [mg kg^{-1}], h_b is the background hazard rate [1/d] (i.e., the control mortality rate, assumed to be constant during the experiment), t is the time [d], and S is the survival probability [unitless].

In addition to GUTS-RED-SD modelling, we also used classic dose-response analysis with conventional probit analysis for the survival data measured at the 48 h and final 96-hour time points using SPSS v.27

(IBM, 2020). Plots were generated using R version 4.0.1 and the 'Ecotox' package (Hlina et al., 2020). Effects on weight change were analysed by calculating the body weight change (BWC) index given as: BWC= (W96h-Wt0)/Wt0; where W96h is the weigh at the end of exposure (after 96 h) and Wt0 is the initial weight at time 0 (start of exposure). Body weight change was analysed using a one-way- ANOVA to assess significance and a post hoc Tukey's test to further identify any significant differences between treatment.

3. Results

3.1. Assay optimisation

3.1.1. Experimental set up

The developed bioassay was relatively easy to set up and to monitor. Compared to other widely used methods such as leaf dip assay, the plates containing the spiked diet used for the exposure are more labour intensive to establish. This increased effort is, however, countered by the ability to easily generate survival data over time through continuous monitoring as needed as input for the GUTS-RED-SD model. Assessment of larval survival for all individuals on a single plate can be done in little more than two minutes, meaning that within an experiment, replicate plates each with 12 individuals for a typical design using a control and six test concentrations can be checked in 12 min. In addition to measuring survival, at each time point assessed, the larvae can be taken from the diet surface, damped to remove any adhered diet or frass and weighed immediately. Weighing larvae is the most time-consuming part of the test, as to carefully remove, clean and weigh all individuals from a plate can take 10 min. This time means it will take approximately one hour to sample and weigh all individuals in a full experiment. Such weight change data is, however, useful for allowing assessment of the sublethal effects of exposure. As such data is not needed for GUTS-RED-SD modelling, this step can be removed for studies focused purely on survival.

3.1.2. Mamestra brassicae survival and growth over the development period

To determine the optimal time for the collection of individuals for use in the subsequent toxicity bioassays, the development of larvae was tracked from hatching to pupation. Larvae reached the correct size for testing (4th instar larva weighing 20 mg ± 10 mg) at a between 12- and 16-days post-hatching (Fig. 1). At this rate of development, if a bioassay was set up at 16 days post –hatching, it would be expected that the larvae exposed on a control diet would reach an average weight of 0.297 g at the completion of the bioassay four days later (20 days post hatching).

3.1.3. Background mortality

Optimised test systems for assessing toxicity either through traditional concentration response modelling or using TK/TD model such as GUTS-RED-SD should, where feasible, achieve a relatively low level of control (background) mortality. Within test guidelines, control survival of $> 80\%$ is usually seen as a suitable maximum for acceptable test performance. For the developed bioassay, 4th instar larva were selected for testing at day 12–16 post hatching, because in initial trials, larvae at this stage showed lower background mortality, compared with earlier instars and also an extended time between moults. To assess the rates of control survival in the assay, the data on survival over time of individuals added to the control treatment of each of the three bioassays can be used. For the three separate studies, the overall rate of survival in controls was 95.8%, 100% and 83.3% respectively. Thus, in all cases, control survival was above the 80% criteria accepted for most regulatory bioassays.

3.1.4. Exposure mortality

The effects of exposure on survival could be readily monitored in time by a visual inspection allowing a high frequency survival checks during the experiment. Use of five-timepoint measurements (including time zero) gave a total of 192 survival checks at 8 concentrations for five time points (= 960 individual assessments per test). The data-set could be used as time discrete data-sets for LC_{50} assessment through Probit analysis (e.g. using the 48 h and 96 h data) for each exposure time or the whole time series data-set could be used as input for GUTS-RED-SD modelling.

The exposure to chlorpyrifos resulted in concentration and time dependent effects on survival in all three experiments. The iterative refinement of the concentration range for testing allowed us to both focus the dose range to generate a more robust assessment of the LC_{50} value from Probit analysis and toxicokinetic and toxicodynamic parameters from GUTS modelling. Dose response curves (Fig. 2), highlight that full mortality at 96 h was found at concentrations above 17.5 mg/kg across all three tests. Based on the three separate experiments, the Probit analyses returned LC_{50} values of 17.1, 20.4 and 10.6 mg/kg for studies 1,2 and 3 at 48 h and of 13.4, 18.3 and 4.7 mg/kg for the three studies at 96 h (Table 1). That the estimated LC_{50} fell within a factor of four for both exposure times indicates a high repeatability of the test, even when different concentrations of the same chemical are tested.

The GUTS-RED-SD models could be fitted for all three experiments using the whole time series data set (full GUTS parameter data including model goodness of fits given in the supplementary material). The GUTS-RED-SD model fits almost all of the data points within the 95% CI indicating a good fit of the measured data to the model predictions (Fig. 3), supported also by the model goodness of fits (see Supplementary material). The no effect concentration (NEC, i.e. median of the threshold distribution, m_w, ± 95% CI) was estimated at 7.4 (7.0–9.6), 15.6 (15.0–15.9) and 3.2 (0.003–5.83) mg/kg for test 1, 2 and 3 respectively. The final test reported a much lower NEC value. Notably, this test also recorded the highest background mortality and did not have an intermediate concentration between 0 and 10.35 mg/kg.

As the GUTS-RED-SD model parameters are derived from the data for the full exposure concentration range over time, they can be used to derive LC_{50} values for any point in time in the experiment. Using the calibrated GUTS-RED-SD model, the LC_{50}s at 48 h were of 13.1, 18.7 and 8.1 mg/Kg respectively in the three test replicates (Table 1). Thus, the LC_{50} values derived from model are within a factor of 2 of those calculated with the discrete data for the 48-hour sampling time for each of the three independent experiments. The LC_{50} values derived from the 48-hour time period from the GUTS-RED-SD parameter vary between the three experiments by a maximum of 2.3 fold and 4 fold at 48 h and 96 h respectively. This consistency between the separate experimental studies indicates that the developed bioassay can provide a repeatable measure of pesticide impacts on survival patterns in the tested lepidopteran species. (Table 2).

3.2. Sub-lethal Effects

To assess sub-lethal effects, larvae were weighed at the start and end of the exposure in the second experiment. This allowed an assessment of the feasibility of adding a sub-lethal effect measurement to the bioassay. Weights at the end of the experiment could only be collected from larvae exposed to chlorpyrifos concentrations up to 16.1 mg/kg due to the complete mortality found for all individuals exposed at the higher tested concentrations. Larvae grew in all measured treatments (Fig. 4). Further, an analysis of variance (ANOVA) of the larval weight change of larvae at each concentration showed no significant variation in the magnitude of weight increase between treatments ($F(4, 107) = 1.66$, $p > 0.05$) (Fig. 4).

4. Discussion

Maintained at a temperature of 20 °C and a 16: 8 light:dark photoperiod, *Mamestra brassicae* eggs have an incubation period of approximately five days. Once larvae hatch they develop through six distinct

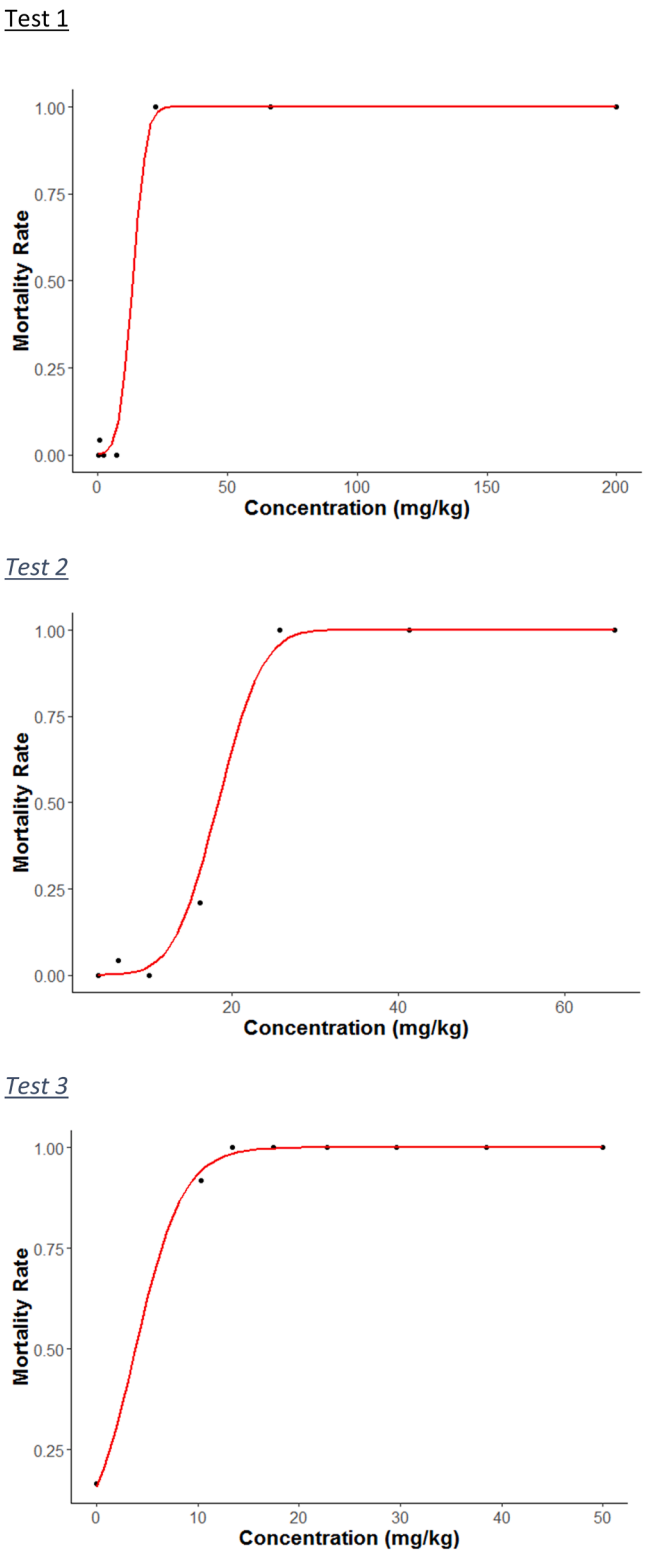

Fig. 2.. Dose response curve for test 1, 2 and 3 at 96 h when modelled by Probit GLM in R. Red line shows mortality rate per concentration.

stages, each of which can be visually recognised by their specific colour changes (Montagne, 1977). Under field conditions, first instar larvae are a yellow/green in colour and have a black head and prothoracic shield (Devetak et al., 2010). These first instar larvae hatch as white on emergence, but then take on a green pigment from their first plant feed (Montagne, 1977). In the culture population, the moth larvae are reared

Table 1
LC_{50} values at 48 h and 96 h following the exposure of *Mamestra brassicae* to an artificial diet spiked with chlorpyrifos, calculated with probit, or derived with the GUTS-RED-SD model. N/A indicates 95% confidence interval could not be calculated.

Test No.	LC_{50} 48 h probit	LC_{50} 48 H GUTS-RED-SD	LC_{50}96 H probit	LC_{50} 96 H GUTS-RED-SD
1	17.1 (N/A - N/A)	13.1 (11.7–15.5)	13.4 (8.3–25)	10.3 (9.5 –12.5)
2	20.4 (18.5–22.5)	18.7 (18–19.6)	18.3 (13.8–28.3)	16.9 (16.4–17.4)
3	10.6 (0.5 – 15.8)	8.1 (5.1–10.2)	4.7 (2 – 6)	5.3 (2.12– 7.8)

on artificial diet from birth; under this regime this pigmentation development is not seen in first to third instar larvae; instead they are cream in colouration. The black head capsule is shed in the 1st instar moult and is replaced by a cream/brown head capsule in all other instars. Third and 4th instar are very similar in appearance, except for the increased prominence of dorsal and sub-dorsal lines. At this stage, larvae are between 7 and 14 mm in length (Sannino and Espinosa, 1999). In our laboratory population, larvae reached third instar at 10 days. The distinct morphological features of the larvae at each instar make it feasible to select individuals at the same life-stage for testing based on simple visual analysis. This standardisation of the selected instars reduces the potential for life-stage specific variation in sensitivity for survival and weight change.

While there are examples of previous toxicity studies with Lepidoptera conducted using artificial diet (Adamczyk et al., 1999; Morimoto et al., 2004; Knight et al., 2001), we believe that the proposed assay is the first to use a design that specifically incorporates the toxicant into the food medium. Although the design does not exclude contact exposure, as the larvae sit on the treated diet, it can provide a greater level of clarity of the nature of the exposure. This covers both the added concentration per unit food source and the possibility to reliably track consumption (e.g. by recording the amount of diet removed by the larvae over the exposure), which is not possible in such a quantitative manner in other tests such as the leaf dip assays. Exposure through both contact and feeding is also a characteristic of existing tests like the leaf dip assay. Hence, the potential for exposure by both routes, but likely dominance of oral exposure, is a consistent feature of both approaches.

From the initial assessments of background mortality rates and development over time (Fig. 1), it was decided to use 4th instar larva for testing. However, given that larvae can develop from egg to pupae within the test system, the overall approach could be used to test any larval instar and indeed for testing effects through the full larval development period. As, however, earlier instars are more prone to deaths arising from handling stress given their small size, the use of the later (larger) instar supports lower background mortality, which is desirable for both concentration response and GUTS modelling. If working with later instars of larger Lepidoptera, one change that can be introduced to the design of is the use of a larger testing surface, such as a six well plate, and a greater amount of added diet to avoid space or food limitation. The potential to test across full larval development could be useful to identify any susceptible stages. It has been proposed that the choice of instar used in bioassays may affect sensitivity to pesticide exposure. Indeed, several studies have highlighted a greater sensitivity to chemicals at lower instars, with the final two stages often being least sensitive to chemical toxins (De Armas et al., 2020; Knight et al., 2001).

Organophosphate insecticides, such as chlorpyrifos, have become the pest management product of choice in many developed and developing countries due to their generic availability, low cost, limited potential for bioaccumulation, and relative environmental persistence (Mitra and Maitra, 2018). In our three bioassays with chlorpyrifos, we found a high degree of repeatability of the results from the exposure,

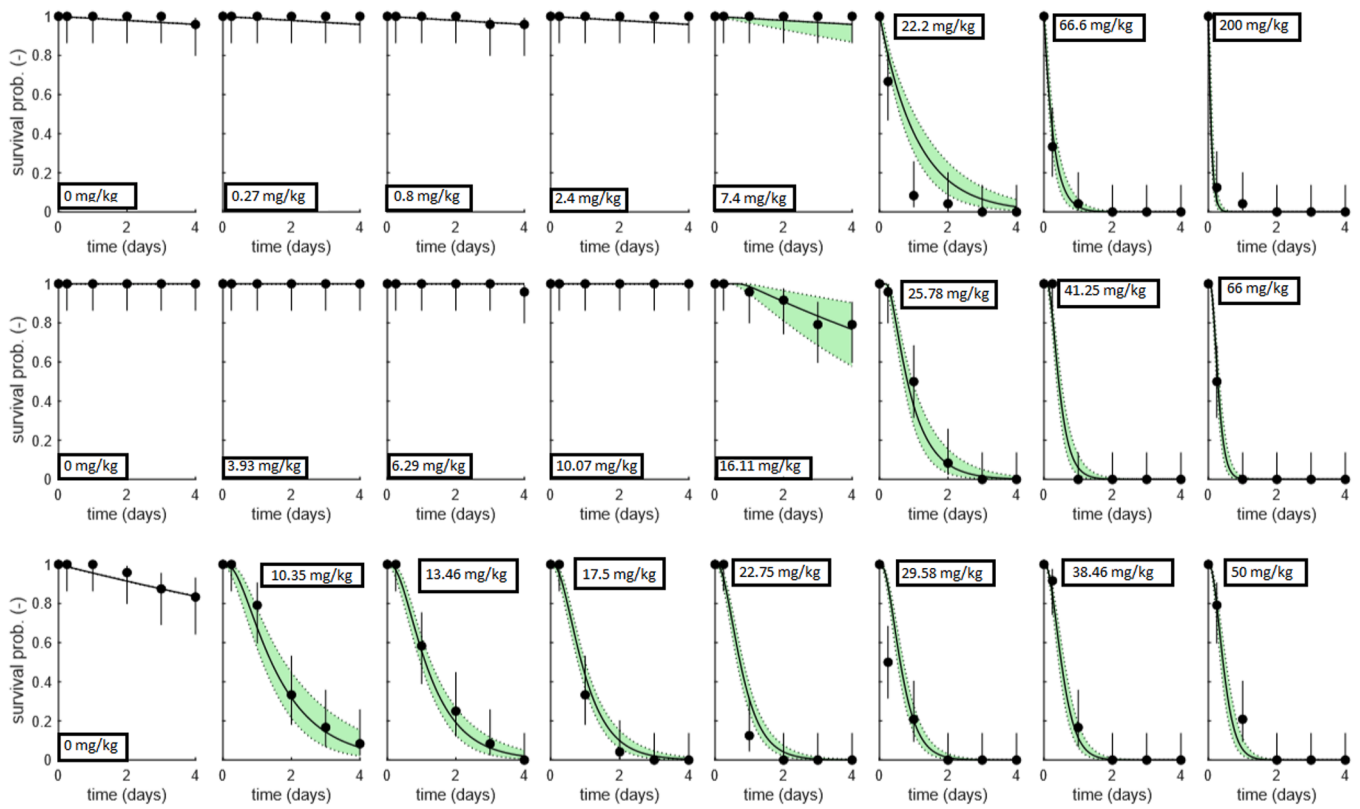

Fig. 3. Observed and simulated survival over time of Mamestra brassicae larvae exposed to an artificial diet spiked with seven concentrations of chlorpyrifos, and a control, in separate laboratory bioassay repeats (first row of plots is test 1, second is test 2 and third is test 3). The points are the measured data (bars show Wilson score confidence), and the lines are the GUTS-RED-SD model simulation (confidence intervals as green area).

Table 2
Parameter values (including 95% Confidence Intervals) of the GUTS-RED-SD model estimated for the 3 assays of Mamestra brassicae exposed to an artificial diet spiked with chlorpyrifos and used to simulate the survival curves presented in Fig. 3. The * denotes where upper CI cannot be calculated because parameter CI has run into a boundary.

Test No.	Background mortality	Dominant rate constant	Median of threshold distribution	Killing rate
	h_b (d^{-1})	k_d (d^{-1})	m_w (mg kg^{-1})	b_w (kg mg^{-1} d^{-1})
1	0.01047	184.2 (13.7–184.2 *)	7.4 (7.0–9.6)	0.061 (0.046–0.084)
2	1 10^{-6}	5.0 (4.0 – 7.4)	15.6 (15.0–15.9)	0.155 (0.108–0.215)
3	0.04445	2.3 (1.1–4.1)	3.214 (0.003 * – 5.836)	0.106 (0.074–0.147)

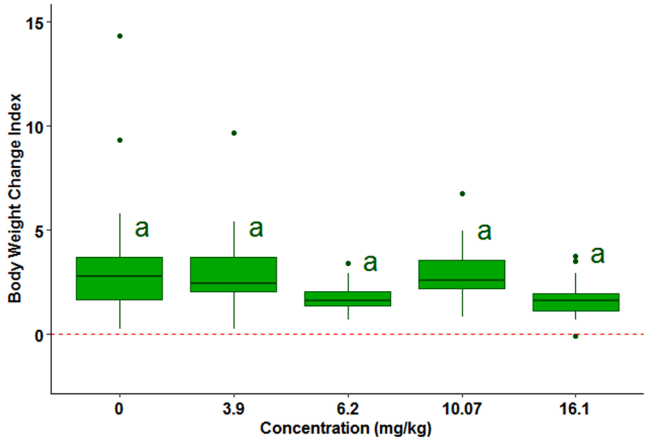

Fig. 4. Box and Whisker plots showing mean (bold line) 75th percentile (upper and lower box limits) and 95% confidence intervals (vertical lines) and outlier values (points) for relative body weight change of Mamestra brassicae larvae exposed for 96 h exposed to different concentrations of chlorpyrifos. A different letter means significant difference (one-way ANOVA test and post hoc Tukey test).

despite the use of different concentration series in each test. Probit analysis of the 48 h time point survival data gave LC_{50} values between 10.6 and 20.4 mg/kg and 96 h values between 4.7 and 18.3 mg/kg. This close agreement verifies the repeatability of the assay, both in term of successful dosing (which could additionally be verified by measurement) and for the larval response. Such demonstration of repeatability is critical to confirming the overall suitability of our approach. Although we developed a novel robust experimental design, our results from the repeated bioassays mirror those from other lepidopteran toxicity tests with organophosphates. Morimoto et al. (2004) using an exposure through artificial diet, found that the lepidopteran *Spodoptera litura* had an LC_{50} of 12.8 mg/L when exposed to another organophosphate, acephate. Greater toxicity of chlorpyrifos than that found here was reported by Kalita et al. (2016) who found an LC_{50} of 3.35 mg/L at 96 h in the silkworm (*Philosamia ricin*) indicating the potential for significant variation in species sensitivity among lepidopterans for this insecticide. Further investigations of such differences in sensitivity would require comparative testing using experimental systems with similar overall designs.

The developed bioassay provided all the required data to calculate toxicity metrics use in risk assessment, as well as the data necessary to calibrate a GUTS model. The differences in toxicity metrics between the

3 tests was low and supporting the robustness of the assay. The use of an LC_{50} value alone to express sensitivity to a given chemical, however, has its limitations. By their nature, LC_{50}s are derived from toxicity data for a single time point. Hence, they do not account for the dynamic/time dimension of the toxic effect. The limitation means that they cannot be compared for assays of different durations (Jager, 2011). TKTD models, such as the GUTS framework, account for the time dimension, and, once calibrated, can be used to calculate LC_{50} values at any time point for regulatory use. In addition, the LC_{50} derived with the GUTS_RED-SD calibrated model are more robust and reliable than the LC_{50} calculated with the classic approach. This is further highlighted by the inability of probit analysis to calculate a confidence interval of the LC_{50} in some tests at 48 and 96 h (Table 1). The GUTS-RED-SD model is parameterised with the mortality data from all time points. Therefore, the outcome of this analysis are less influenced by any specific value than toxicity metric (e.g. LC_{50}s) generated with data from only a single (i.e. the final) time point. These results support the advantages of TKTD modelling over traditional probit analysis to obtain LC_{50} values. Further, all of the GUTS-RED-SD model parameters (i.e. m_w and k_d) were all in the same range when comparing the three tests. The similarity of these values highlights the repeatability and reliability of the bioassay to generate the required data to calibrate a robust GUTS-RED-SD model. A further advantage of the GUTS-RED-SD model is that the derived parameters provide a wider insight into the nature of the toxic effect than is possible from simple concentration response model fitting.

In all 3 tests, the GUTS-RED-SD model provided a very good simulation of the data (Fig. 3), indicating the data generated by the experimental design were of good quality, and once calibrated, the model could be used to predict new scenarios, e.g. time varying concentrations. Among the estimated parameters, the threshold parameter m_w (i.e. the NEC) is intrinsically not time dependent, and, therefore, provides a more valuable and comparable perspective of hazard than LC_{50} values which vary with time (Fig. S2). The dominant rate constants "k_d" approximate the toxicokinetic elimination rate "k_e" if the damage recovery is fast (i.e., TD). Alternatively, if the damage recovery is slow relative to the toxicokinetic elimination, the k_d will approximate the damage repair rate "k_r" (i.e. TD). If both dominant rate constants are of similar size, then k_d will represent the one compartment approximation of the `true' two-compartment TK and TD behaviour (Jager et al., 2011). Here the estimated k_d is not fast (except for test 1, but the CI is large so not well characterized for this test) nor slow. Such intermediate values indicate that chlorpyrifos is excreted (or metabolised) at a certain rate, and/or the damages are repaired at a certain rate as well.

The developed bioassay procedure can also be modified for the analysis of effects such as weight change over a time course, an advantage over previous lepidopteran testing. However, in this study, our choice of chemical and tested concentrations may not be particularly suited to this type of analysis due to its lethality to *M. brassicae* larvae.

The weight change data did not highlight any significant differences in the body weight change index between 0 and 96 h. However, the median weight change of control larvae exceeded that of all chlorpyrifos treatments. Weight change associated with exposure to chlorpyrifos could occur for a number of reasons. The first, could be a change in metabolic activity caused by exposure leading to an increase in maintenance costs to eliminate the chemical and repair any resulting damage due to the toxicant effects. Such trade-offs between growth and maintenance are included within the wider concept of Dynamic Energy Budget Theory (DEB)(Kooijman and Bas 2009; Jager and Zimmer, 2012). A second theory is that chlorpyrifos could have an anti-feedant effect. Previous studies have found that aside from the high invertebrate toxicity of organophosphates, compounds from this class can have strong repellent effects (Kalita et al., 2016). However, given that the lowest median weight change occurred at 6.2 mg/Kg, but not at the intermediate concentrations, further work would be needed to link feeding or resource allocation changes to effects on larval growth.

The potential to obtain sub-lethal data for effect on weight can allow the further development of the GUTS-RED-SD based TKTD survival models into full life-cycle Dynamic Energy Budget toxicity (DEBTox) models. It is hoped that with modifications to the bioassay, this kind of study can be undertaken as a DEBTox modelling approach would allow predictions to be made on the TKTD effects of chemical exposure on multiple traits relating to survival, growth, and potentially, development and reproduction. As this bioassay is optimised for use on larvae, effects on reproduction are not possible to obtain within the assay system. It may, however, be possible to extend the length of the test to get the growth data up to pupation and calibrate only the growth part of a DEB model. To measure reproduction, a different exposure strategy for adult moths would be needed. This could potentially include spiking the 10% honey solution that is used for adult culturing to allow dietary exposure.

5. Conclusion

Lepidopterans are a speciose rich and diverse order of insects that can be both beneficial and pest organisms in agriculture. Currently, there is a relative lack of knowledge of the effects of pesticides on life-cycle endpoints and the nature of the biological pathways that underlie TKTD traits in lepidopteran species. This paucity of information is due in part to the lack of a bioassay method capable of generating necessary data to apply to TKTD models, such as GUTS-RED-SD. Here we develop and test a novel bioassay, suitable for toxicity testing for the effects of pesticide exposure in time on a larval life-stage. The new bioassay is able to generate highly repeatable results over a longer range of time and with a high frequency of survival assessment for multiple individuals across multiple exposure levels. For testing, here we used *Mamestra brassicae* larvae due to their ease of rearing and rapid reproduction rates. However, it is expected that, with diet optimisation, the bioassay would be suitable for alternative lepidopteran species. While tested for single chemicals, the overall method could be applied for mixture toxicity testing as the chemicals are added into the diet while in a liquid state, allowing multiple chemicals to be added in a single treatment. This method proved appropriate for dosing, given the known stability of chlorpyrifos. However, this method may need to be verified for chemicals where temperature stability is low or not known. The design means it would be possible to adapt the protocol to allow for pulsed exposures, by removing larvae from spiked plates and transferring them to clean artificial diet, potentially extending the test duration to cover exposure at different larval stages. Overall, the bioassay system presents a robust and reliable approach to measuring the potential impacts of chemical exposure in lepidopteran larvae and could be deployed at scale for high throughput testing.

CRediT authorship contribution statement

Claire Badder: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Sylvain Bart:** Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Alex Robinson**: Conceptualization, Resources, Investigation. **Helen Hesketh**: Conceptualization, Validation, Resources. **Peter Kille**: Resources, Supervision, Funding acquisition. **David J. Spurgeon**: Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: David Spurgeon reports financial support was provided by UK Research and Innovation. Claire Badder reports financial support was provided by UKRI.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.114504.

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