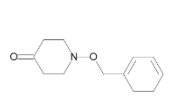
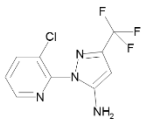
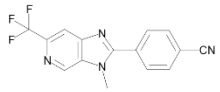
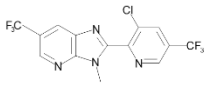
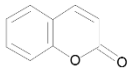
Parent compounds				
A	**B**	**C**	**D**	**Coumarin**
				

Fate of synthetic chemicals in the agronomic insect pest *Spodoptera littoralis:* experimental feeding-contact assay and toxicokinetic model.

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Abstract

Insecticides prevent or reduce insect crop damage, maintaining crop quality and quantity. Physiological traits, such as an insect's feeding behaviour, influence the way insecticides are absorbed and processed in the body (toxicokinetics), which can be exploited to improve species selectivity. To fully understand the uptake of insecticides, it is essential to study their total uptake and toxicokinetics independently of their toxic effects on insects. We studied the toxicokinetics (TK) of insecticidally inactive test compounds incorporating agro-like structural motifs in larvae of the Egyptian cotton leafworm (*Spodoptera littoralis*), and their distribution across all biological matrices, using laboratory experiments and modelling. We measured *Spodoptera* larval behaviour and temporal changes of whole-body concentrations of test compounds during feeding on treated soybean leaf disks and throughout a subsequent depuration period. Differences in the distribution of the total quantities of compounds

were found between the biological matrices leaf, larva, and feces. Rate constants for uptake and elimination of test compounds were derived by calibrating a toxicokinetic model to the whole-body concentrations. Uptake and elimination rate constants depended on the physicochemical properties of the test compounds. Increasing hydrophobicity increased the bioaccumulation potential of test compounds. Incomplete quantities in larval matrices indicated that some compounds may undergo biotransformation. As fecal excretion was a major elimination pathway, the variable time of release and number of feces pellets led to a high variability in the body burden. We provide quantitative models to predict the toxicokinetics and bioaccumulation potential of inactive insecticide analogues (parent compounds) in *Spodoptera*.

Keywords: agrochemistry, exposure, depuration, absorption, excretion.

Introduction

As both the global human population as well as global demand for agricultural products continue to increase, there is a continued need to develop effective methods and tools for regenerative agriculture (Roser et al. 2013). Insecticides have become an important part of this, but it is vital that their ecological impact is minimal and well understood. Combined effects of climate change and human population growth make even more important to ensure food availability and to avoid crop losses due to pests (Haile 2000, Meyers and Kalaitzandonakes 2015). Estimated global annual crop losses were around 52 % in 2015 (BVL, 2015), with pests and diseases accounting for a significant proportion of it. Insects, including beetles, aphids, and caterpillars, accounted for about 15 % of total losses. Effective pest management, including insecticide use, is essential to maintain and improve crop yields by keeping pest populations below economic thresholds (Buntin 2000, Haile 2000, Tonnang et al. 2022). Insecticides also help to reduce post-harvest losses caused by pest damage during storage and transport. Understanding of the mechanisms contributing to these losses and of the efficacy of insecticides is therefore crucial to develop effective strategies for sustainable agriculture and ensure food security (Carvalho 2006, Seufert et al. 2012).

Insecticide exposure can have an impact on biological performance parameters of exposed individuals, from sub-lethal effects to mortality (Müller et al. 2017, Müller et al. 2019). Performance parameters such as feeding behaviour, body mass, and reproductive output are indicators of individual fitness of organisms (Gutsell and Russell 2013, Schuijt et al. 2021). When those insecticide effects are intended, we view them as aspects of efficacy. When similar effects occur in non-target organisms, we view them as unintended toxicity. Making insecticides more selective, i.e., optimising for efficacy, but with minimum environmental toxicity, is key for sustainable food

production and biodiversity protection. Various methods in chemistry design are established to reduce the number of compounds to be synthesised and tested (Dudek 2006, Gichere 2021). This aims to improve efficacy against targets, while gaining selectivity on non-targets. Selectivity can be achieved through favourable environmental fate and bioavailability in target pests, as well as differentiating toxicokinetics and toxicodynamics.

The toxicokinetics (TK) of insecticides, which include processes such as uptake, distribution, biotransformation, and excretion, are important drivers of the biologically effective dose, which impacts on efficacy or toxicity. Species-specific differences in TK between target and non-target insects are particularly important as they may help to maximise efficacy against pests while minimising adverse environmental impact. Studies of TK across and within species (Ashauer et al. 2012, Nyman et al. 2014) could provide valuable insights for designing selectivity and therefore developing safer and more efficient insecticides.

Studies of TK in non-target organisms, and the corresponding experimental designs, are well established in environmental toxicology (Rubach et al. 2010, Nyman et al. 2014). However, studies on TK in target insect species are rare in the scientific literature. As a result, we up to date do not have available published standardised experimental design and data analysis workflow for toxicokinetics of chemicals in target insects. Hence, this study aimed to develop such an assay combined with chemical analysis and toxicokinetic modelling. This was evaluated for potential use in chemistry optimisation.

The larvae of the Egyptian cotton leafworm, *Spodoptera littoralis*, are severe agronomic chewing pests that can infest more than 80 different crops (De Groote, Kimenju et al. 2020) and are representative of foliar Lepidopteran pests. Our primary objective was to develop a *Spodoptera* bioassay combined with chemical quantification, to characterise the fate of chemicals in fed leaf disks and insects. The assay also measured behavioural responses to assess exposure-induced changes using image analysis. Our secondary objective was to build a species- specific toxicokinetic (TK) model capturing internal compound concentrations over time.

Materials & Methods

Test compounds

Four representative insecticidally inactive test compounds (log P range 1.43-3.57 and molecular mass 146-303 g/mol, Figure 1, Table 1) were synthesized in-house (≥ 95% purity). They incorporate structurally scaffolds or fragments with some degree of insecticide-likeness. Additionally, coumarin [CAS 91-64-5, (≥ 95% purity, Merck KGaA, Darmstadt, Deutschland), Figure 1, Table 1] was used as a reference for extraction and as a method standard. All compounds were dissolved at 2000 mg/L in water containing 15 % acetonitrile (ACN) (gradient

grade for analytics 99.9 %) as solvent. A high dosing should also enhance the possibility of detecting putative metabolites. In addition, this rate did not show any adverse effects on larval performance parameters.

Plants

Soybean plants, *Glycine max* (L.) Merr cv. Toliman, were used in the *S. littoralis* feeding-contact assay. Four soybean seeds were germinated and grown per pot (Ø 6.5 cm) filled with white peat growth medium. Plants were grown in a greenhouse under controlled conditions (14 h light (27 Lux., 22 °C) and 8 h dark (18 °C) cycle, 65 ± 5 % relative humidity (RH)) and used in the assay after 14 days of germination. Two leaf disks (Ø 20 mm) were cut from two fully developed true leaves and stored on wet filter paper to minimise desiccation.

Insect

Egyptian cotton leafworm (*Spodoptera littoralis;* BOISDUVAL, 1833) were reared in the laboratory under standardised conditions (23 ± 1 °C, 55 ± 10 % RH), including an in-house artificial diet for both adults and larvae. This laboratory strain had not been exposed to insecticides.

Larvae stages were synchronised by transferring the derived second larval stage (L2) from artificial diet to an empty Petri dish (Ø 12 cm). The dish contained only dry filter paper and was covered with a cotton filter. Within two hours, the larvae moulted into the early third larval stage (L3).

***Spodoptera* bioassay**

The assay combined an exposure phase of 24 h followed by a depuration phase of 24 h under standardised conditions (25 ± 1 °C, 55 ± 10 % RH, 16-hour light/8-hour dark cycle, Figure 2). *S. littoralis* larvae were exposed to treated leaf disks, reflecting a preventative bioassay with oral and contact uptake of test compounds.

Before exposure, 50 µl of test solutions, containing 0.1 mg of test compounds, were evenly distributed on the leaf disks (Ø 20 mm) by shaking for 20 seconds at 300 rpm using a pipetting robot (Fluent® Automation Workstation, Tecan Group Ltd, Männedorf, Switzerland). The control treatment received a 15 % ACN-water solution. After 30 min evaporation of test solution, leaf disks were placed in a 12-well microtiter plate laid out with moist filter paper to maintain humidity (12-MTP, FalconTM, Northfield, Minnesota, USA).

One freshly moulted *S. littoralis* larva (L3) was placed on each leaf disk in a microtiter plate then covered with a transparent foil with evaporation holes. Larvae were exposed to treated leaf disks for 24 h, then transferred to a microtiter plate containing untreated leaf disks for a 24 h depuration period (Figure 2).

S. littoralis larvae samples were collected during the exposure period (T0-24 h) and the depuration period (T24-48 h). As a reference, 3 g leaf samples (n=133 pooled leaf disks) were collected from a parallel assay without larvae, and therefore no real mass balance can be established. Samples were taken after 0, 1, 5, 24, 25, 29, 48 hours (soybean samples: pooled leaf disks per time point, n=3; *S. littoralis* samples: one larva per time point, n=12). Feces samples (pooled feces pellets per larva, n=12) were collected at the end of the exposure (period T0-24 h) and depuration phase (period T24-48 h). All samples were transferred to 2.5 ml tubes (MP Biomedicals™ FastPrep-24™ 5G, Lucerne Chem AG, Lucerne, Switzerland) and immediately frozen at -80 °C to stop metabolism (Figure 2).

Larval performance parameters

Larval behaviour and performance parameters (larval size, food consumption, excretion, and movement) were observed by images acquisition in 1-hour-intervals during exposure period (Figure 2). Larval size (segmented pixels converted to mm^2), food consumption (proportion % leaf area) conversion of food to feces (count of feces pellets), and movement (comparison between frames) were recorded visually using methods described previously (Sadeghi-Tehran et al. 2017, Sadeghi-Tehran et al. 2019).

Residue measurements

Sample preparation

The same chemical analytical method was applied to all biological matrices to quantify compound concentrations, but extraction differed for larvae, feces, and leaf disks. As all samples were processed as a total mass of the given biological matrix (leaf disk, larva, feces), it is therefore not possible to distinguish whether the detected compound(s) were absorbed internally or adsorbed on the surface (Figure 2).

For each larva, the total body wet weight (wwt) was measured after thawing using a Sartorius-balance (BCE124I-1S Entris® II, Data Weighing Systems, Inc., Wood Dale, IL, USA). Larvae and feces samples were both homogenised using a macerator (MP Biomedicals™ FastPrep-24™ 5G, Lucerne Chem AG, Lucerne, Switzerland) with a ceramic ball (Ø 6.35 mm, MP Biomedicals™ zirconium oxide-coated beads, Lucerne Chem AG, Lucerne, Switzerland). Next, 500 µl ACN were added to each sample, before shaking for 3 h at 300 rpm and 20 °C using an Eppendorf ThermoMixer® (Merck & Cie, Schaffhausen, Switzerland). After shaking, samples were centrifuged at 9000 rpm for 2 min. Preparations of leaf samples involved addition of 30 ml ACN and a cleaning buffer step before centrifugation (buffer I 8g mixture: 450 g $MgSO_4$, 115 g sodium acetate). After centrifugation leaf and feces samples were filtered through a 0.20 µm pore size filter (CHROMAFIL®Xtra PET-20/13, Macherey-Nagel

GmbH & Co.KG, Düren, Germany). Supernatants (150 µl) of each sample were transferred to analytical glass vials, with a 200 µl glass insert (Vials N11, with 0.2 ml insert, Macherey-Nagel GmBH & CO.KG, Düren, Germany).

Chemical analysis

Ultra High-Performance Liquid Chromatography Mass Spectrometry (UHPLC-MS) was performed using ACN as solvent. Spectra for parent compounds and their putative metabolites were recorded from all samples on a Mass Spectrometer (Xevo TQ-XS Triple Quadrupole Mass Spectrometer) from Waters Corporation equipped with an Electrospray Ionization Source (ESI) (Figure 2).

Parent compound and metabolites in samples were chromatographically separated on an Acquity Iclass Plus system with an Acquity UPLC High Strength Silica (HSS) column (T3, 2.1 x 30 mm, particle size 1.8 µm) using a water solution (A) (90 % water, 10 % methanol, and 0.1 % formic acid) and (B) ACN (0.1 % formic acid). Samples were measured in gradient elution mode with fluctuating flow rates. The gradient flow consisted of following steps: initial flow rate of 1.0 ml/min of 80 % A/20 % B until 0.10 min, then from 0.10 min to 0.20 min to 25 % B, followed by a flow rate change to 0.750 ml/min till 1.20 min with 30 % B, from 1.20 min to 1.45 min to 100 % B, then until 1.45 min to 20 % eluent B, and finally, from end of the run at 2 min with a flow rate of 0.050 ml/min and 50 % B in an isocratic mode. Column temperature was maintained at 60 ± 5 °C, and sample injection volume was 2 µl. For MS detection conditions, the desolvation Gas Flow was set at 1000 L/h at temperature of 500 °C. The flow rate of the cone gas was set at 150 L/h, with capillary voltage of 3 kV, source temperature of 150 °C, and cone voltage ranging from 15 to 60 V. Detection of parent compound was performed by single ion recording (SIR) in a Mass Range of 120 to 1000 Da. The parent compound was quantified using a calibration series.

Toxicokinetic modelling

We determined uptake and elimination rates by calibrating a one-compartment first-order toxicokinetic (TK) model to the measured concentration in the larvae and leaf disks (internal concentration data).

The toxicokinetic model can be represented mathematically as:

(1)

$$\frac{d\,C_{\mathrm{i}}(\mathrm{t})}{dt} = k_{\mathrm{in}} * C(\mathrm{t})_{\mathrm{leaf}} - k_{\mathrm{out}} * C_{\mathrm{i}}(\mathrm{t})$$

where $C_i(t)$ represents the internal concentration of the parent compound [$mg_{compound}/mg_{wet\_weight}$] in and on the organism (whole body residue), t is time (h), $C(t)_{leaf}$ is the external concentration [$mg_{compound}/mg_{leaf}$] in and on the leaf disk, k_{in} [$mg_{leaf}/(mg_{wet\_weight}*h)$] the uptake rate constant and k_{out} [1/h] is the elimination rate constant.

Equation 1 was applied separately for each compound, yielding compound specific uptake and elimination rate constants. The ordinary differential equations (ODEs) were implemented using the MATLAB (Version R2021a) Build Your Own Model (BYOM v60_beta5) platform (https://www.debtox.info/byom.html) and we used the maximum likelihood estimation with a normal likelihood function for model calibration by minimising the likelihood difference between measured and modelled internal body concentrations (Jager and Ashauer 2018b). Confidence intervals were calculated for the uptake and elimination rate constants with likelihood profiling, with an upper limit of 100 is set by the BYOM platform, which can be equated with infinity (Table 1, Figure S4).

Bioaccumulation factor

The bioaccumulation factor (BAF) is the ratio of the concentration of the test compound within the organism in comparison to the external source at steady state, specifically the concentration in *Spodoptera* larvae compared to the treated soybean leaf disk. The BAF can be calculated as the ratio between the uptake rate constant (k_{in}) and elimination rate constant (k_{out}).

(2)

$$\mathrm{BAF} = \frac{k_{\mathrm{in}}}{k_{\mathrm{out}}}$$

A BAF value can indicate whether the concentration of the test compound in the organism is higher than the concentration in the external source. To calculate the confidence intervals of the BAF, the model was run at a constant concentration (set to 1) until steady-state and the resulting confidence interval of the internal concentration equals the confidence interval of the BAF (Ashauer et al. 2010).

Results

Larval performance parameters

Larval performance parameters (food consumption, excretion, larval size, movement) did not deviate from untreated controls during the 24 h exposure (Figure 3, Figure 4). Larvae of all treated groups continued feeding without detectable influence of day and night shift. At the end of the 24 h exposure period all larvae had consumed almost the entire leaf disk (Figure 3, Figure S1). The average size of a larva after 24 hours of exposure was 55.9

193 mm^2 (Figure 4, Figure S2). On average, the larvae transformed one leaf disk (Ø 20 mm) into 33 feces pellets
194 (Figure 3, Figure S3). Feces dropping usually started about 4 to 5 hours after infestation with larvae (Figure S3).
195 The movement of the *S. littoralis* larvae between image frames was constant throughout the exposure (Figure 3).
196 This means that exposure to test compounds did not disrupt normal food consumption, defecation, growth, and
197 movement pattern of L3-larvae.

198 **Compound quantities in all compartments**

199 Quantities of compound A, C and D on the leaf disks were consistent during 24 h of the exposure period, and
200 therefore demonstrated stable exposure profiles (Figure 5 a). In contrast, quantities of compound B decreased by
201 more than 50 %, as compared to the dosed amount, during the exposure period, from 0.1 mg to 0.05 mg of the
202 parent compound per leaf disk sample within the first five hours of the experiment. Coumarin quantities started to
203 decline after one hour down to 0.005 mg at 24 h (Figure 5 a).

204 The time-course of compound quantities in larvae differed between the compounds and during exposure and
205 depuration periods (Figure 5 b). Quantities of compounds A, C and D increased during the 24 h exposure period
206 and decreased during depuration period (Figure 5 b). Compounds C and D reached maximum levels (median 100
207 % of treated leaf disk) of the parent compound quantity in larval bodies after 24 h exposure, whereas compound
208 A reached only 30 % (median) after 24 h and compound B reached 30 % already after 5 h. Coumarin showed
209 maximum compound quantity levels of 60 % (median) within the larva after 1 h of exposure, which decreased to
210 15 % at the end of the period (Figure 5 b).

211 Feces samples represent the sum of all feces pellets of individual *S. littoralis* larvae sampled after exposure (T0-
212 24 h) or depuration (T24-48 h) respectively. At the end of the exposure period, the fecal quantities of compounds
213 A and coumarin were with about 60 % (median) of the dosed compound the highest detected fractions, whereas
214 compound B, C, and D demonstrated quantities of parent compound in the range of 15-25 % (median) (Figure 5
215 c). Chemical quantities in feces pellets remained below 5 % (median) in the depuration period for all compounds,
216 except compound D. Here the quantities in feces increased up to 30 % (median) of parent compound (Figure 5 c).

217 **Toxicokinetic model**

218 Overall, the compound treatments showed clear differences in the concentrations of parent compounds in the larvae
219 and the resulting uptake and elimination rate constants and bioaccumulation factors (Table 1). The TK model fits
220 the concentration at 24 h and the elimination period rather than the concentration in the first 5 h (Figure 6). All
221 compounds and coumarin showed higher uptake rate constants than elimination rate constants, resulting in

bioaccumulation to reach concentrations in larvae above the levels in leaf disks (Table 1, Figure S4). The TK model curves (Figure 6) further highlights differences between the compounds, especially in their time-course of uptake and depuration. In the exposure phase, the concentrations at the first sampling point were already at a similar concentration level to those at the following sampling point (5 h). The model calibration of compound A, B, and C resulted in parameters hitting a boundary (k_{in} at upper limit) (Figure S4). Coumarin showed a rapid uptake, followed by a steep decline due to the declining exposure (Figure 6) and the model parameters converged with confidence intervals, which were well-identified (closed parameter likelihood plot Figure S4). The model of compound D also converged with closed confidence intervals (Figure S4).

Discussion

In a first investigative step performance parameters were analysed for potential effects of chemical exposure (Müller and Müller 2015). All larvae exhibited normal behavior during the exposure period of the experiments because no changes of behavioral patterns of larvae compared to control groups were detectable (Figure 4, Figure S1-3). We can therefore conclude that behavior is not predominantly responsible for detected differences in compound quantities in biological matrices (Kingsolver and Huey 2008, Ankley et al. 2010, Gergs et al. 2015).

During the exposure period, we found substantial variation of quantities in larval bodies over time (Figure 5 b, Figure 6). As measurable excretion of compounds begins with the first dropping of feces pellets after an average of four to five hours of feeding and the feces pellets contain a substantial amount of test chemicals, the highest variation in body tissue concentration variation was observed in this time frame (Figure 5, Figure 6). Interestingly, the variation in compound quantities in larval bodies is also reflected in the increased variation of quantities in feces (Figure 5 c). Whether this was caused by different exposure or elimination should be investigated in more detail in future studies. Compound uptake into larval bodies might lead to high biotransformation, resulting in low quantities in feces. This illustrates the interaction between bioavailability, uptake, and excretion. The substantial and quick elimination through fecal egestion is also an indication that only a limited quantity of compounds could be absorbed systemically into larval bodies. Some of the compounds might be passing the gut without being absorbed.

For stable chemicals one can expect 100 % recovery of the parent compound across all matrices and the apportionment between the different matrices describes the fate of chemicals over time in plant and larval tissues in the given assay. Compound A, C and D demonstrated chemical stability on the leaf disk. Maximum levels of compound quantities were observed in *S. littoralis* bodies once the entire leaf disks were eaten up after 24 h, but

with low quantities in feces. This suggests that the test compounds remained unchanged in these plant and insect matrices. In contrast, compound B reached the maximum quantities in larval bodies at 5 h. At 24 h quantities in larval bodies and feces were lower than 20 % (median) of the exposure dose. Abiotic degradation could be excluded by control experiments without larva, leaving only biotransformation as a likely cause of low recovery. The decline in compound quantities in leaf disks and larvae could potentially be explained with biotransformation (Figure 5).

Toxicokinetic (TK) modelling is a valuable tool for understanding species differences in uptake kinetics, bioaccumulation, and the role of metabolism. Standardised assays improve the reliability and reproducibility of data (Bonta 2002, Jager and Ashauer 2018a). TK modelling enables comprehensive assessments of non-target risks and informed environmental management decisions (Ashauer and Escher 2010, Hommen et al. 2015).The observed differences in compound quantities in larval bodies are explainable by different kinetic rate constants for uptake and elimination. Uptake rate constants varied by more than ten orders of magnitude between compounds, while excretion rate constants varied by only a factor of about two (Figure 6, Table 1). This was already demonstrated earlier with other organisms, such as annelids (Belfroid et al. 1993, Šmídová et al. 2021). TK models have been shown to be capable to predict the toxicokinetics of compounds in a range of organisms (Nyman et al. 2014) (Table 1). Here we show that the TK modelling approach that is well established in environmental toxicology and the risk assessment of non-target species risk assessments can be adapted to target organisms.

The current experimental design cannot distinguish between contact and oral uptake of test compounds. Therefore, we modelled the uptake over time without discriminating between both principal absorption routes. Nevertheless, varying absorption routes could be a major differentiator between target and non-target species due to different biology, e.g., feeding types. Beside food consumption, contact absorption by crawling on the leaf disk can contribute to substantial uptake (Chown and Nicolson 2004, Beran and Petschenka 2022). This uptake would then definitively lead to systemic exposure. The bioassay could be adapted to separate contact or oral exposure, with an assay design that uses only oral intake (forced feeding) or pure contact from treated surface (Hamby et al. 2013, Balabanidou et al. 2018, Denecke et al. 2018, Arlos et al. 2020).

After analyzing the experimental uptake curves and their variability, we recommend to sample with higher frequency during the beginning of exposure period (≤ 5 h), and at the beginning of the depuration period (Figure 6, Figure S4). This would help to better understand this critical part of the toxicokinetics, specifically the curvature

of the modelled internal concentration. More data points would help to achieve also better and more robust model fits.

The simple first-order one-compartment TK model employed here is unable to explain all patterns in the body tissue concentration data because it cannot differentiate between exclusive gut passage and systemic uptake. At least those chemicals that show degradation in the insect should have had some systemic uptake unless the gut microbiome also contributes to biotransformation. Unfortunately, the TK model (Table 1, Figure, 6, Figure S4) does not capture well the observed uptake of compound A, B, and C, presumably due to the variability in the onset of the dropping of fecal pellets as this appears to be the most important elimination pathway. Nevertheless, under these assay conditions the TK model captures the basic patterns of TK in *S. littoralis* L3 larvae for five different test compounds (Table 1). Whilst the TK model generally reflected the compound concentrations within larval bodies, we also measured compound quantities in feces, but this information was not considered by the model. Additionally, the dilution of internal concentrations due to larval growth over time was not considered. *S. littoralis* larvae increased their body mass by a factor of 4 during the exposure, as they ate the entire leaf disk. Both, excretion via feces and growth dilution could be added to the toxicokinetic model. Here we wanted to apply the simplest model first to demonstrate the suitability of the method in general. More complex models could be considered in future studies if appropriate data can be generated. A combined understanding of the organisms' biology (performance parameter, total quantities of compound in insect body or excretion product) and more frequent measurements of internal concentrations could help to better understand the putative starting point of detoxification due to biotransformation.

Many phytophagous species, especially pest species such as Lepidoptera, possess a variety of enzymatic degradation pathways and detoxification mechanisms, such as excretion, to prevent bioaccumulation (Dow 1992, Schulz 1998, Roberts and Hutson 1999, Perić-Mataruga et al. 2019).

As molecules with higher Log P values tend to have a greater affinity for biological membranes (Hofstetter et al. 2018), their bioaccumulation potential is higher (Hawker and Connell 1985, Esser 1986). In our study, the bioaccumulation factor (BAF) generally increased with increasing hydrophobicity, confirming this rule (Table 1), with the exception of coumarin, which is putatively metabolized. The residence time of a parent compound within a larval body not only can significantly influence the toxic effects on a Lepidopteran pest species, but has been shown to raise resistance potential due to enzymatic processes in pest species (Wing et al. 1998, Siegfried and

Scharf 2001). In our experiments, all compounds were completely eliminated from the larval bodies within the depuration period (Figures 5, Figure 6).

In conclusion, we successfully developed and implemented a bioassay which characterised the fate of synthetic chemicals in plant, insect, and excretion in an agronomic relevant Lepidopteran pest. This study highlights the complexity of compound uptake, excretion, biotransformation, bioaccumulation, and biological response in *Spodoptera littoralis* larvae. These insights will support chemistry optimisation i.e., the identification of more selective insecticides which are more effective against target pests and which possess minimal environmental toxicity. the identification of more selective insecticides which are more effective against target pests, and which possess minimal environmental toxicity. Future studies could apply a similar experimental design and data analysis approach to other important Lepidopteran pest species. This experimental approach could contribute to a more comprehensive understanding of the uptake and excretion of agrochemicals.

TK models are a valuable approach to understand internal compound concentrations in target organisms and once parameterized, predict exposure under different conditions. Nevertheless, the total amounts in all compartments of the bioassay should be observed to obtain a realistic mass balance of the compound. Since we did not estimate the contribution of biotransformation within plant or insect, future research should include biotransformation measurements. Biotransformation could have a major impact on performance parameters, exposure, depuration, bioaccumulation, and finally toxicity (Ashauer et al. 2012, Rosch et al. 2016). It provides a better understanding of pest-specific patterns, which further supports the development of effective pesticides with the lowest possible environmental impact (Nyman et al. 2014).

Declarations' section

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Authors contribution

CIR: Formal analysis, Investigation, Methodology, Project administration, Software – Programming, Visualization, Writing – original draft, Writing – review & editing

RA: Conceptualization, Software – Programming, Supervision, Visualization, Writing – review & editing

BIE: Supervision, Writing – review & editing

KH: Formal analysis, Methodology, Software – Programming, Validation

MM: Conceptualization, Formal analysis, Supervision, Writing – review & editing

PST: Formal analysis, Methodology

NS: Formal analysis, Methodology, Software – Programming, Validation, Visualization

AB: Conceptualization, Methodology, Project administration, Supervision, Visualization, Writing – review & editing

All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on request.

353 **References**

354 Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount DR, Nichols JW, Russom CL,
355 Schmieder PK, Serrrano JA, Tietge JE, Villeneuve DL. Adverse outcome pathways: a conceptual framework
356 to support ecotoxicology research and risk assessment. Environ Toxicol Chem. 2010;29(3):730–741.
357 Arlos MJ, Schurz F, Fu Q, Lauper BB, Stamm C, Hollender J. Coupling river concentration simulations with a
358 toxicokinetic model effectively predicts the internal concentrations of wastewater-derived micropollutants in
359 field Gammarids. Environ Sci Technol. 2020;54:1710–1719
360 Ashauer R, Escher BI. Advantages of toxicokinetic and toxicodynamic modelling in aquatic ecotoxicology and
361 risk assessment. J Environ Monit. 2010;12:2056–2061.
362 Ashauer R, Caravatti I, Hintermeister A, Escher BI. Bioaccumulation kinetics of organic xenobiotic pollutants in
363 the freshwater invertebrate Gammarus pulex modeled with prediction intervals. Environ Toxicol Chem.
364 2010;29:1625–1636.
365 Ashauer R, Hintermeister A, O'Connor I, Elumelu M, Hollender J, Escher BI. Significance of xenobiotic
366 metabolism for bioaccumulation kinetics of organic chemicals in Gammarus pulex. Environ Sci Technol.
367 2012;46:3498–3508.
368 Balabanidou V, Grigoraki L, Vontas J. Insect cuticle: a critical determinant of insecticide resistance. Curr Opin
369 Insect Sci. 2018;27:68–74.
370 Belfroid A, Vanwezel A, Sikkenk M, Vangestel K, Seinen W, Hermens J. The toxicokinetic behaviour of
371 chlorobenzenes in earthworms (Eisenia andrei): experiments in water. Ecotoxicol Environ Saf. 1993;25:154–
372 165.
373 Beran F, Petschenka G. Sequestration of plant defence compounds by insects: from mechanisms to insect–plant
374 coevolution. Annu Rev Entomol. 2022;67:163–180.
375 Bonta J. Offender Risk Assessment: Guidelines for Selection and Use. Crim Justice Behav. 2002;29:355–379.
376 Buntin GD. Techniques for evaluating yield loss from insects. Biotic Stress and Yield Loss. 2000;23.
377 Carvalho F. Agriculture, Pesticides, Food Security and Food Safety. Environ Sci Policy. 2006;9:685-692.
378 Chown S, Nicolson S. Insect Physiological Ecology: Mechanisms and Patterns. Oxford University Press. 2004.
379 Denecke S, Swevers L, Douris V, Vontas J. How do oral insecticidal compounds cross the insect midgut
380 epithelium? Insect Biochem Mol Biol. 2018;103:22-35.
381 Dow JA. pH gradients in lepidopteran midgut. J Exp Biol. 1992;172:355-375.

Esser HO. A review of the correlation between physicochemical properties and bioaccumulation. Pestic Sci. 1986;17:265-276.

Gergs A, Kulkarni D, Preuss TG. Body size-dependent toxicokinetics and toxicodynamics could explain intra- and interspecies variability in sensitivity. Environ Pollut. 2015;206:449-455.

Gutsell S, Russell P. The role of chemistry in developing understanding of adverse outcome pathways and their application in risk assessment. Toxicol Res. 2013;2:299-307.

Haile FJ. Drought stress, insects, and yield loss. In: Biotic Stress and Yield Loss. 2000:117-134.

Hamby KA, Alifano JA, Zalom FG. Total effects of contact and residual exposure of bifenthrin and λ-cyhalothrin on the predatory mite Galendromus occidentalis (Acari: Phytoseiidae). Exp Appl Acarol. 2013;61:183-193.

Hawker DW, Connell DW. Relationships between partition coefficient, uptake rate constant, clearance rate constant and time to equilibrium for bioaccumulation. Chemosphere. 1985;14:1205-1219

Hofstetter S, Beck A, Trapp S, Buchholz A. How to design for a tailored subcellular distribution of systemic agrochemicals in plant tissues. J Agric Food Chem. 2018;66:8687-8697.

Hommen U, Schmitt W, Heine S, Brock T, Duquesne S, Manson P, Meregalli G, Ochoa-Acuña H, van Vliet P, Arts G. How TK-TD and Population Models for Aquatic Macrophytes Could Support the Risk Assessment for Plant Protection Products. Integr Environ Assess Manag. 2015;12.

Jager T, Ashauer R. How to Evaluate the Quality of Toxicokinetic-Toxicodynamic Models in the Context of Environmental Risk Assessment. Integr Environ Assess Manag. 2018;14:604-614.

Jager T, Ashauer R. Modelling survival under chemical stress: A comprehensive guide to the GUTS framework.

Kingsolver JG, Huey R. Size, temperature, and fitness: Three rules. Evol Ecol Res. 2008;10:251-268.

Meyers WH, Kalaitzandonakes N. World Population, Food Growth, and Food Security Challenges. In: Food Security in an Uncertain World. Vol. 15. Emerald Group Publishing Limited. 2015:161-177.

Müller T, Müller C. Behavioural phenotypes over the lifetime of a holometabolous insect. Front Zool. 2015;12(Suppl 1):S8.

Müller T, Prosche A, Müller C. Sublethal insecticide exposure affects reproduction, chemical phenotype as well as offspring development and antennae symmetry of a leaf beetle. Environ Pollut. 2017;230:709-717.

Müller T, Römer CI, Müller C. Parental sublethal insecticide exposure prolongs mating response and decreases reproductive output in offspring. J Appl Ecol. 2019;56:1528-1537.

Nyman AM, Schirmer K, Ashauer R. Importance of toxicokinetics for interspecies variation in sensitivity to chemicals. Environ Sci Technol. 2014;48:5946-5954.

Perić-Mataruga V, Ilijin L, Mrdaković M, Todorović D, Prokić M, Matić D, Vlahović M. Parameters of oxidative stress, cholinesterase activity, Cd bioaccumulation in the brain and midgut of Lymantria dispar (Lepidoptera: Lymantriidae) caterpillars from unpolluted and polluted forests. Chemosphere. 2019;218:416-424.

Roberts T, Hutson D. Imidacloprid. Metabolic Pathways of Agrochemicals—Part 2: Insecticides and Fungicides. Journal Abbreviation. 1999.

Rosch A, Anliker S, Hollender J. How biotransformation influences toxicokinetics of azole fungicides in the aquatic invertebrate Gammarus pulex. Environ Sci Technol. 2016;50:7175-7188.

Roser M, Ritchie H, Ortiz-Ospina E. World population growth. Published online at OurWorldInData.org.

Rubach MN, Ashauer R, Maund SJ, Baird DJ, Van den Brink PJ. Toxicokinetic variation in 15 freshwater arthropod species exposed to the insecticide chlorpyrifos. Environ Toxicol Chem. 2010;29:2225-2234.

Sadeghi-Tehran P, Virlet N, Sabermanesh K, Hawkesford MJ. Multi-feature machine learning model for automatic segmentation of green fractional vegetation cover for high-throughput field phenotyping. Plant Methods. 2017;13:103.

Sadeghi-Tehran P, Virlet N, Ampe EM, Reyns P, Hawkesford MJ. DeepCount: In-Field Automatic Quantification of Wheat Spikes Using Simple Linear Iterative Clustering and Deep Convolutional Neural Networks. Front Plant Sci. 2019;10:1176.

Schuijt LM, Peng FJ, van den Berg SJP, Dingemans MML, Van den Brink PJ. (Eco)toxicological tests for assessing impacts of chemical stress to aquatic ecosystems: Facts, challenges, and future. Sci Total Environ. 2021;795:148776.

Schulz S. Insect-Plant Interactions− Metabolism of Plant Compounds to Pheromones and Allomones by Lepidoptera and Leaf Beetles. Eur J Org Chem. 1998;1998:13-20.

Seufert V, Ramankutty N, Foley JA. Comparing the yields of organic and conventional agriculture. Nature. 2012;485:229-232.

Siegfried BD, Scharf ME. Mechanisms of organophosphate resistance in insects. In: Biochemical Sites of Insecticide Action and Resistance. 2001:269-291. Springer.

Šmídová K, Svobodová M, Hofman J. Toxicokinetics of hydrophobic organic compounds in oligochaeta: A critical review. Environ Pollut. 2021;289:117743.

Tonnang HE, Sokame BM, Abdel-Rahman EM, Dubois T. Measuring and modelling crop yield losses due to invasive insect pests under climate change. Curr Opin Insect Sci. 2022;50:100873.

Wing KD, Schnee ME, Sacher M, Connair M. A novel oxadiazine insecticide is bioactivated in lepidopteran larvae. Arch Insect Biochem Physiol. 1998;37:91-103.

Table 1: Toxicokinetic model parameters (uptake and elimination rate constants, bioaccumulation factors) for *Spodoptera littoralis* (L3) larvae and chemical descriptors (log P, molecular weight) of tested compounds.

Compound	Log P	Molecular weight (g/mol)	Uptake rate constant (k_{in}) ($mg_{leaf}/(mg_{wet\_weight}*h)$)	Elimination rate constant (k_{out}) (1/h)	Bioaccumulation factor (BAF) ($mg_{leaf}/mg_{wetweight}$)
A	1.58	205.25	2.63 [0.61;>100]	2.24 [0.68;>100]	1.17 [0.78;1.58]
B	1.50	262.62	8.89 [3.31;>100]	5.43 [2.10;72.45]	1.64 [1.29;1.99]
C	2.24	302.25	4.86 [1.89;>100]	1.68 [0.63;45.21]	2.89 [2.26;3.85]
D	3.57	280.68	2.79 [1.26; 6.79]	0.73 [0.33;1.74]	3.82 [3.53;4.92]
Coumarin	1.43	146.14	18.71 [10.64;72.71]	4.52 [2.58;17.11]	4.16 [2.64;3.83]

Figure 1: Structure of test compounds (A-D), and coumarin. (Measured log P and molecular mass shown in Table 1.)

Figure 2: Toxicokinetic assay design: (a) Individual *Spodoptera littoralis* larvae were exposed to compound treated leaf disks. Feeding contact assay with a 24 h exposure period (including imaging) followed by a 24 h depuration period. (b) Schematic sample preparation. Biological samples were macerated. After extraction and centrifugation, the clear supernatant was used for residue measurements by Ultra-High Performance Liquid Chromatography-Mass Spectrometry. Figure created with BioRender.com.

Figure 3: Image frames of the *Spodoptera littoralis* assay: Representative example of a single larva feeding pattern on a leaf disk observed in the toxicokinetic assay over 24 h of exposure (see also Fig. S1).

Figure 4: Physiological parameters of *Spodoptera littoralis* after 24 hours exposure period, created by image analysis. Consumed leaf area (Proportion = pixel per mm^2), number of feces pellets, and larval size, shown for all test groups (control, test compounds A-D, coumarin). Boxplots show interquartile ranges, medians (black lines), and means (×). Whiskers not exceeding 1.5 × of the interquartile range extend to the maximum and minimum. Individual data points (n = 12), including outliers, are shown as circles. Figures created using R (version 3.5.3; R Core Team, 2020).

Figure 5: Compound quantities (mg) quantified per **a)** soybean leaf disk, **b)** *Spodoptera littoralis* larvae (body) and **c)** feces pellets. Exposed larvae fed on treated leaf disk for 24 hours, immediately after exposure interval larvae

were transferred and fed on non-treated leaf disk for a follow-up depuration time of 24 hours. A separate bioassay without larvae feeding on the leaves was used for the leaf disk (a) measurements of the compound. Boxplots show interquartile ranges and medians (black lines). Whiskers not exceeding 1.5 × of the interquartile range extend to the maximum and minimum. Outliers are shown as circles. Figures created using R (version 3.5.3; R Core Team, 2020).

Figure 6: Leaf disk concentrations and body concentrations in *Spodoptera littoralis* larvae exposed to five test compounds. Exposed larvae fed on treated leaf disks for 24 hours. Followed by transfer to untreated leaf disks for a depuration period of 24 hours. (a) TK-Model for *Spodoptera littoralis:* parent compound uptake (mg/mg wet weight) and elimination (mg/mg wet weight). The model curve represents the best-fit parameter values (Table 1) and 95 % confidence limits (dotted) of model fit represented by the lines. Dots indicate measured data. (b) Exposure scenarios in feeding contact assay: soybean leaf disk concentrations during exposure and depuration time (green line). Created using MATLAB (Version R2021a, Build Your Own Model).