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ORIGINAL ARTICLE

Effects of pyrethroid and organophosphate insecticides on reared black soldier fly larvae (*Hermetia illucens*)

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Abstract Black soldier fly larvae (*Hermetia illucens*) receive growing interest as a potential alternative animal feed source. These insects may be exposed to insecticide residues in the rearing substrate. This study aimed to investigate the effects of six different pyrethroid and organophosphate insecticides on this insect species' performance. The toxicity of two "model" substances for each of these classes (cypermethrin; pirimiphos-methyl) was quantified, with and without the synergist piperonyl butoxide (PBO). Critical effect doses corresponding to 10% yield (CED10) for cypermethrin (0.4 mg/kg) and pirimiphos-methyl (4.8 mg/kg) were determined. The addition of PBO to cypermethrin enhanced its relative potency with a factor 2.6. These data were compared against the relative toxicity of two analogue substances in each class (permethrin, deltamethrin; chlorpyrifos-methyl, malathion). Results suggest that exposure to concentrations complying with legal limits can cause significant reductions in yield. Exposure to multiple substances at lower concentrations resulted in negative additive and synergistic effects. Of the tested substances, deltamethrin was most toxic, causing 94% yield at 0.5 mg/kg. Analytical results suggested that transfer of tested substances to the larval biomass was substance- and concentration-specific, but appeared to be correlated to reduced yields and the presence of PBO. Transfer of organophosphates was overall low (<2%), but ranged from 8% to 75% for pyrethroids. Due to very low limits in insect biomass (~0.01 mg/kg), high transfer may result in noncompliance. It is recommended that rearing companies implement lower contractual thresholds, and that policymakers consider adjusting legally allowed maximum residue levels in insect feed.

Key words feed; insects; pesticides; rearing; substrate

Introduction

Larvae of the black soldier fly (BSFL, *Hermetia illucens* (L.); Diptera: Stratiomyidae) and other insect species are increasingly used for food and feed purposes (Barragan-Fonseca *et al.*, 2017; Wang & Shelomi, 2017; Bosch *et al.*, 2019; Bessa *et al.*, 2020). However, during mass-rearing, these insects may be exposed to insecticide residues in their diet or "substrate," which is composed of organic residues originating from agriculture or other sources. Exposure to insecticide residues may result in lethal and sublethal effects (Desneux *et al.*, 2007; Guedes *et al.*, 2011) as well as bioaccumulation of the insecticidal substances or derived metabolites in the larvae, thereby posing a safety risk for the animal or human who would be consuming the contaminated

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insect-derived products (EFSA, 2015; Meyer *et al.*, 2021). In a previous study, a significant increase in mortality and reduction in total yield was observed when 7-d-old BSFL were exposed to the pesticides spinosad (2.0 mg/kg wet weight) and cypermethrin (CYP, 0.3 mg/kg wet weight) over a rearing period of 7 d (Meijer *et al.*, 2021). Mean concentrations in the larvae were relatively low at 0.12 mg/kg for spinosad and 0.12 mg/kg for cypermethrin, which suggested that bioaccumulation of the parent compounds was not observed for these substances. However, the maximum residue level (MRL) for "terrestrial invertebrate animals," including reared insects for feed or food, is set at the default of 0.01 and 0.05 mg/kg for spinosad and cypermethrin, respectively (Regulation (EC) No 396/2005). As such, even minimal transfer rates from substrate to insect biomass could lead to noncompliance of the produced larval biomass when used in feed or food. Varying levels of insecticides in collected or reared edible insects have recently been reported in, for instance, several African countries (Labu *et al.*, 2022; Poma *et al.*, 2022), Thailand (Kanthawongwan *et al.*, 2019), Belgium (Poma *et al.*, 2017), and Canada (Kolakowski *et al.*, 2021)—suggesting that the presence of insecticide residues in insect products marketed for human consumption are a cause for some concern. Therefore, these results warranted additional research on the effects of insecticide residues on BSFL. The possibility of cumulative or synergistic effects if residues of multiple substances are present in the feed materials is of particular concern (EFSA, 2020; Geissen *et al.*, 2021). The effects of multiple combined insecticides are also pertinent for environmental risk assessment. For instance, a recent field study by Brühl *et al.* (2021) found residues of up to 16 pesticides to be present in samples of flying insects collected in nature conservation areas adjacent to agricultural land.

Organophosphate and pyrethroid insecticides are among the most used synthetic insecticidal compound classes in the European Union (EU) (Eurostat, 2021). These two classes encompass a large variety of substances that are well studied in the context of their effects on pest species (Elliott *et al.*, 1978; Hirano, 1989; Siegfried & Scharf, 2001). However, results of such studies may not necessarily translate well to the conditions of commercial mass-rearing settings of nontarget insect species. Both pyrethroids and organophosphates target the nervous system of insects (Sparks *et al.*, 2020). Pyrethroids do so primarily by interfering with the voltage-gated sodium channels, thereby causing rapid paralysis ("knockdown"), which results in desiccation or predation under field conditions (Khambay & Jewess, 2005; Soderlund, 2010; Wakeling *et al.*, 2012), but often recovery is observed after a few days in laboratory settings, even at high doses (Khambay & Jewess, 2005). Pyrethroids are generally classified into two main groups, as based on the biological response that is associated with the absence ("Type I," e.g., permethrin [PER]) or presence ("Type II," e.g., CYP) of an α-cyano group in the molecule (Casida *et al.*, 1983; Khambay & Jewess, 2005). Type I pyrethroids induce rapid hyperactivity leading to knockdown at relatively low concentrations, but recovery may occur, whereas for type II, the onset of symptoms is slower, but the kill-rate is higher and recovery is less likely to occur (Khambay & Jewess, 2005). Primary detoxification of pyrethroids is generally occurring through hydrolytic and oxidative cleavage of the ester bond by cytochrome P450 monooxygenase and esterase enzymes—while specific secondary metabolic products and associated pathways are more dependent on the species in question (Bradbury & Coats, 1989; Scott, 2001; Khambay & Jewess, 2005; Bhatt *et al.*, 2020).

The synergist PBO (PBO) is often used in commercial formulations in conjunction with a pyrethroid, with the aim to increase the efficacy of the pyrethroid (Tozzi, 1999) and—in some species—even to bypass pyrethroid resistance (Young *et al.*, 2005; Romero *et al.*, 2009; Bingham *et al.*, 2011). PBO synergizes pyrethroids by inhibiting activity of P450 monooxygenase and esterase enzymes, thereby preventing detoxification (Snoeck *et al.*, 2017). In a previous study (Meijer *et al.*, 2021), an increase of mortality by 12% and reduction of yield of 25%, compared to the control, was found for BSFL which were exposed to the pyrethroid CYP at 0.3 mg/kg (which is the MRL for maize). Addition of PBO (6.0 mg/kg) resulted in 22% mortality and 59% reduction in growth. When CYP was spiked at a lower concentration of 0.1 mg/kg, no significant effects on survival and growth were observed, nor was this the case when PBO (2.0 mg/kg) was added.

The mode of action of organophosphates is inhibition of the acetylcholinesterase (AchE) enzyme, resulting in hyper-excitation of post-synaptic cholinergic neurons and eventually insect death (Siegfried & Scharf, 2001). The toxicity of some organophosphates is dependent on oxidative bioactivation by P450 monooxygenases (Siegfried & Scharf, 2001). Detoxification and metabolization of organophosphates has been linked to P450 monooxygenases, glutathione S-transferases, and hydrolytic enzymes (Siegfried & Scharf, 2001). In a previous study, no significant effects were observed for the organophosphate chlorpyrifos at concentrations of 0.05 (MRL in wheat, Regulation (EC) No 396/2005) and 0.5 mg/kg (10× MRL) (Meijer *et al.*, 2021). Purschke *et al.* (2017) investigated the bioaccumulation and effects

on BSFL biomass of the organophosphates chlorpyrifos, chlorpyrifos-methyl (CM), and pirimiphos-methyl (PM). They concluded that bioaccumulation was low, and they found no significant effects on BSFL biomass at 2.5 mg/kg of each of these substances. A negative cross-resistance relationship has been suggested between pyrethroids (that are detoxified by P450s) and certain organophosphates (that are activated by P450 enzymes) in a variety of insect species (Cilek *et al.*, 1995; Scott, 1999; Kolaczinski & Curtis, 2004; Smith *et al.*, 2019; Yunta *et al.*, 2019). Furthermore, inhibition of P450 enzymes by PBO has, in some species, been found to reduce the toxicity of some organophosphates (diazinon, chlorpyrifos, azinphos-methyl), while the toxicity of organophosphates that did not require P450-induced bioactivation (dichlorvos) was unaffected by the addition of PBO (Ankley & Collyard, 1995). As such, we hypothesized that the addition of PBO to a P450 bioactivated organophosphate (i.e., PM) would reduce mortality.

The aim of this study was to gather more insight on the effects of pyrethroid and organophosphate insecticides on the survival and total biomass (yield of BSFL reared for food and feed to. The focus was on the effects of the selected model substances CYP (pyrethroid) and PM (organophosphate) in isolation, in combination, and synergized by PBO, on BSFL insect yield, as well as on substance transfer from substrate to the BSFL biomass. Additional analogue substances in the same respective insecticide classes were included in the research to compare against the model substances and assess potential cumulative effects when combined. These analogues were permethrin and deltamethrin (PER and DEL, pyrethroids) and malathion and chlorpyrifos-methyl (MAL and CM, organophosphates). We believe this to be the first in-depth study to investigate the effects of combined insecticides on BSFL reared for food and feed.

Materials and methods

The study design was adapted from the World Health Organization's (WHO) guidelines for testing mosquito larvicides (World Health Organization, 2005). In summary, 7-d-old *Hermetia illucens* (BSF) larvae were exposed to pyrethroids, organophosphates and combinations in the feed substrate for a period of 7 d. Substances were tested individually in a variety of concentrations and in several combinations. In total 50 treatments were tested (including the controls) in three sequential experiments. Results of each experiment informed the design of the next experiment. Although terrestrial invertebrates such as BSFL are not within the scope of EU legislation on the protection of animals used for scientific purposes (Directive 2010/63/EU), experiments were conducted sequentially to reduce the number of animals required, in accordance with ethical animal testing principles. An additional small-scale experiment to assess the effects of insecticide residues on younger, 1-d-old BSFL, was also conducted. The methodology and results of this separate experiment are presented in Appendix D.

Selection of treatments

The objective of Experiment 1 was to gather preliminary data on the effects of the two model substances on BSFL, in terms of survival and yield, and on the synergizing potential of PBO. Based on the significant reduction in BSFL survival and yield observed for CYP at 0.3 mg/kg in a previous study (Meijer *et al.*, 2021), and the fact that the MRL for this substance in wheat is 2.0 mg/kg, it was decided to select 2.0 mg/kg as the base concentration in Experiment 1. Wheat is a common BSFL substrate ingredient (Scala *et al.*, 2020; English *et al.*, 2021), and 2.0 mg/kg is the highest MRL for CYP, which also applies to barley, oat, rice, and rye (Regulation (EC) No 396/2005). In addition, concentrations of a factor 4 times higher (8.0 mg/kg) and lower (0.5 mg/kg) were tested. Additional treatments with PBO were included for the 0.5 and 2.0 mg/kg treatments, at ratios of 1 : 1, 1 : 10, and 1 : 20. The MRL for PM in wheat is 5.0 mg/kg; therefore, this MRL concentration as well as 1/2 and 2 times the MRL were included. PM at the highest concentration (10.0 mg/kg) was tested in conjunction with PBO at ratios of 1 : 1 and 1 : 4.

Experiment 2 aimed to gather more data needed to construct concentration/response (C/R) curves for the effects of tested substances on BSFL yield. The choices of treatments in Experiment 2 were partly based on the results of Experiment 1. In addition, two treatments were included to test the effects of CYP in combination with PM, at concentrations equal to their respective MRLs, and 1/10 thereof. Finally, these two combined treatments were each repeated with the addition of PBO at a ratio of 1 : 20 for the concentration of CYP.

Experiment 3 aimed to assess the effects effectiveness of two analogues of each of the model substances CYP and PM, in isolation and in combinations, on BSFL yield. Based on the results of Experiments 1 and 2, calculations for a C/R curve were performed to determine the concentrations of CYP and PM for which yield would be 50% of the control value (YC50). These YC50 concentrations were validated in Experiment 3 for the model substances, as well as for the four selected analogues. Two treatments

Table 1 Overview of concentrations of substance(s) tested in each of the three experiments (Experiment 1, 2, or 3). Intended concentrations in mg/kg feed substrate on wet weight basis.

Treatment	Substance(s) per treatment	Experiment 1	Experiment 2	Experiment 3
Control	Blank control	n/a	n/a	n/a
	Solvent control (ACN)	n/a	n/a	n/a
	Piperonyl butoxide (PBO)	40.0	40.0	40.3
Single substance	Cypermethrin (CYP)	0.5	1.0	1.6
		2.0	4.5	0.5
		8.0		
	Deltamethrin (DEL)			1.6
				0.5
	Permethrin (PER)			1.6
				0.5
	Pirimiphos-methyl (PM)	2.5	7.5	10.1
		5.0		3.4
		10.0		
	Chlorpyrifos-methyl (CM)			10.1
				3.4
	Malathion (MAL)			10.1
				3.4
Synergized (+PBO)	CYP + PBO	0.5 + 0.5	0.2 + 4.0	1.3 + 26.0
		0.5 + 5.0	1.0 + 20.0	
		0.5 + 10.0		
		2.0 + 2.0		
		2.0 + 20.0		
		2.0 + 40.0		
	PM + PBO	10.0 + 10.0		
		10.0 + 40.0		
Combined substances	CYP + PM		0.2 + 0.5	2.0 + 5.0
	CYP + PM + PBO		0.2 + 0.5 + 4.0	
			2.0 + 5.0 + 40.0	
	CYP + DEL + PER			0.5 + 0.5 + 0.5
	CYP + DEL + PER + PBO			0.5 + 0.5 + 0.5 + 31.4
	PM + CM + MAL			3.4 + 3.4 + 3.4
	PM + CM + MAL+ PBO			3.4 + 3.4 + 3.4 + 40.3

n/a: nonapplicable.

containing a model substance as well as its respective analogues, each substance at 1/3 of the YC50, were used to determine the potential cumulative effects of these insecticides on BSFL. This was repeated with the inclusion of PBO, and all substances were tested individually at 1/3 of the respective YC50 of the model substance. Larval samples collected in Experiment 3 were analyzed to determine concentrations in this matrix, and assess transfer from the substrate to the larvae. Analytical details are provided in Chemical analyses section.

Table 1 shows the intended concentrations (mg/kg) of substances in treatments in each of the three experiments.

Substrate preparation

Details of the used insecticide reference standards are shown in Table A1. The intended concentrations and corresponding volumes of insecticidal substances in the feed were calculated using the final wet weight of feed

substrate, as provided to the larvae. All substances were dissolved in acetonitrile (ACN; Ultra LCMS, Actu-All Biochemicals, Oss, The Netherlands), or acetone (ACE) in case of CM (2000 μg/mL in both cases). As such, in each experiment, an ACN solvent control was used in addition to a blank control. The ACN volume added to these controls was equal to the sum of the highest volume of ACN that was used as a solvent to a treatment in the respective experiment.

The spiking of the substrate in the three experiments was largely based on the method described by Mulder *et al.* (unpublished). In short, for each treatment, a slurry was created, consisting of 100 g dry feed (Meelfabriek de Jongh, Steenwijk, The Netherlands) and ∼200 mL methanol (MeOH, Ultra LCMS, Actu-All Biochemicals, Oss, The Netherlands), to which the insecticides used in each treatment were added. The slurries were mixed in a glass beaker using a 300 W Hand Mixer (Philips, Amsterdam, The Netherlands). The slurries were subsequently deposited into an open aluminium tray and left overnight in a fume hood for the organic solvents (MeOH, ACN, ACE) to evaporate. The next day, the material was weighed to determine weight loss. For each replicate, 17.5 g of dried feed was deposited into a replicate container. The replicate containers were cylindrical (diam. 100 mm, height 40 mm) and contained a circular area (diam. 40 mm) in the center of the lid, which had a fine mesh to allow for ventilation (SPL Life Sciences Co., Ltd., Gyeonggi-do, South Korea). The treatments were performed in triplicate. From each batch, several aliquots were taken for later analysis to verify the concentration and homogeneous distribution of the spiked substance(s) in selected cases. At the facility where the experiment took place (Bestico B.V., Berkel en Rodenrijs, The Netherlands), 32.5 mL tap water was added to the 17.5 g dry feed in each replicate container to have 50 g wet feed at a water : feed ratio of 65 : 35.

Experimental procedures

Each treatment was performed with three replicates. At the start of all three experiments (experimental day 1), 50 individual 7-d-old larvae were counted and weighed for each replicate. The larvae were acquired from the rearing facility of Bestico B.V. If the weight of a batch of 50 larvae was outside of the 95% confidence interval in that experiment, the larvae were discarded and 50 new larvae were counted. Due to their minute size and the large number of larvae required for each experiment, it was not feasible to weigh each larva individually at the start of the experiment. The larvae were added to the containers, which were subsequently distributed over stacked trays. Climate chamber conditions were set at 28 °C and 60% RH, similar to Meijer *et al.* (2021). On experimental day 6, 2.5−5.0 mL tap water was added to most replicate containers to offset moisture loss, in line with the standard company mass-rearing procedure. Containers in which the feed was assessed to be still very wet, or in which substantial mold growth was observed, did not receive additional water. On experimental day 8, the containers containing larvae and residual material were weighed. The larvae in each replicate container were counted and moved from the experimental containers into separate containers using metal tweezers and subsequently washed and dried as described in Meijer *et al.* (2021). The larvae were then weighed again to determine the yield. The larvae were killed by freezing and kept frozen (at −18 °C) until subsequent chemical analyses. The experimental containers holding the remaining residual material were discarded. Primary variables used for calculations of experiments were the number of larvae on day 8 as a percentage of the number ($n = 50$) of larvae with which the experiment was started ("survival" [%]); the total larval biomass on day 8, after washing of the larvae ("yield" [g]); and the mean individual larval weight ("mean larval weight" [mg]). Mean individual larval weight was calculated by dividing total yield by the number of larvae surviving. The term "yield" is a function of the measures survival and mean individual larval weight, which are measures of lethality and sublethality, respectively.

During the experiments, it was observed that the variation in larval size in certain treatments (across all replicates in each treatment) was higher than in others. The mean individual larval weight, estimated based on total larval weight and total number of larvae, was considered an inadequate measure to highlight this variation. Therefore, each larva of the first replicate of each treatment in Experiment 3 was weighed individually to gain insight in the variation in individual larval weight.

Statistical analysis

Differences between treatments Initial statistical analyses were performed in SPSS Statistics for Microsoft Windows 6 (version 25.0.0.2, IBM Corp., Armonk, NY, USA). Nonparametric statistical tests were used because the number of replicates per treatment ($n = 3$) did not warrant tests on conformity to a distribution type.

Based on results from previous studies, PBO in isolation does not affect BSFL yield and survival. The treatment containing only PBO was therefore classified as a

control for initial statistical analysis. First, it was verified whether the distribution of three variables survival, yield, and mean larval weight was the same across the 3 control treatments (blank, solvent, and PBO) that were used in every experiment, using Kruskal–Wallis tests ($\alpha = 0.05$). If differences were not significant ($P > 0.05$), the values of the three control treatments were pooled ($n = 9$) for subsequent statistical analyses. Second, a Kruskal–Wallis test was used to test whether the distribution of all treatments and the pooled controls was the same across the three variables of interest. If differences were significant ($P \leq 0.05$), a *post hoc* Mann–Whitney *U* test was used to compare each treatment to the pooled controls in an experiment, applying an α of 0.01 to account for multiple comparisons.

Regression curves were plotted for the yield of the treatments from both Experiments 1 and 2 containing (1) CYP; (2) CYP + PBO at a CYP : PBO ratio of 0.05; and (3) PM. This was done using the regression curve-fit command of SPSS. Yield was considered the primary response variable for the population under commercial mass-rearing conditions, instead of the traditional lethal dose for the population (LC). The type of curve that best fitted the respective data (based on R^2 and F values) was selected, and the YC50 was calculated. This preliminary YC50 was used to guide the concentrations to be spiked in the final third experiment, as discussed in Selection of treatments section.

Benchmark dose derivation C/R curves were plotted, and calculations were performed, including all data from all tested treatments in the three experiments with yield as the response variable. This was done for the two model substances CYP and PM, and CYP in conjunction with PBO, using the analyzed concentrations of substances. These calculations were performed using the PROAST software package version 70.5 (RIVM National Institute for Public Health and the Environment, Bilthoven, The Netherlands) for the R programming language version 4.2.0 (EFSA, 2017; Slob, 2018; RIVM, 2022). This software package allows for derivation of the Benchmark dose (BMD), which is an estimation of the dose or concentration associated with a specific response—taking statistical uncertainties into account. The software's quality estimate is the Akaike Information Criterion (AIC): a lower value denotes a higher quality. One of two nested exponential models numbered E3-CED ($y = a \times \exp(b x\hat{}d)$ with $a > 0$, $d > 0$) and E5-CED ($y = a \times [c - (c - 1) \exp(-b x\hat{}d)]$ with $a > 0$, $b > 0$, $c > 0$, $d > 0$) was used, recommended for the single continuous data sets in this study. The difference between the E3 and E5 model is that the c parameter is only used for curves that level off: the E3 model is essentially the same as the E5 model, but with c equaling infinity. Biologically, the curve leveling off at the bottom suggests that there is a certain minimum response value above 0. Either of the two models with the lowest AIC value, that is, the best fit to the data, was used for further data analysis (EFSA, 2017). The models were plotted using a critical effect dose (CED) of 10% yield as the benchmark, with a 90% confidence interval (CI). Outliers identified by the software, based on Grubb's test (RIVM, 2022), were removed. The PROAST software allows the user to appoint a factor as a covariate in the model, to examine to what extent the C/R in certain subgroups differ from each other, using the aforementioned AIC as a criterion (EFSA, 2017; RIVM, 2022). Due to differences in total yield of the controls between the three experiments in this study, in particular, Experiments 1 and 2 compared to Experiment 3, the experiment number was used as a covariate in these analyses.

The observed yields of BSFL biomass when exposed to CYP and PBO at a ratio of 1 : 20 in the feed substrate were also plotted in terms of the relative potency factor (RPF), compared to CYP without PBO. This was done in PROAST using the dedicated E5 model for RPFs. This model is equivalent to the E5-CED model, with the difference that the different CEDs (related to different subgroups) are expressed as RPFs relative to the CED of one of the subgroups (e.g., the reference chemical) (RIVM, 2022).

Finally, the estimated effect size for the MRL applicable for wheat of each substance was determined. MRLs have been established in EU legislation for all tested substances (Regulation (EC) No 396/2005). These limits apply to dry wheat as placed on the market (Article 18 of Reg. 396/2005), while the concentration in the feed as provided to the insects in this study was based on the wet weight, that is, with 65% added water. For this reason, the estimated concentration/effect relationship was corrected for this percentage with a 35% concentration factor (Article 20 of Reg. 396/2005). The estimated effect size was calculated by model averaging "bootstrapping" with 200 runs, as recommended (RIVM, 2022). These results are presented by providing the estimated effect size in % reduction in yield, followed by the 5th and 95th percentiles of the bootstrap runs in brackets.

Chemical analyses

Feed samples from all treatments were analyzed with a validated and accredited liquid chromatography-mass spectrometry (LC-MS/MS) based analytical procedure

after a QuEChERS extraction to verify the spiked concentration. In addition, homogenous distribution of the spiked substances in the diet of Experiment 1 was verified by analyzing $n = 10$ aliquots for selected treatments that were assumed to be representative to validate the spiking method, also with LC-MS/MS as mentioned before. This was done for the treatments containing CYP (8.0 mg/kg), CYP and PBO (0.5 and 0.5 mg/kg), PM (2.5 mg/kg), and PM and PBO (10.0 and 10.0 mg/kg respectively). Finally, selected larval samples from Experiment 3 were analyzed by a gas chromatography-mass spectrometry (GC-MS/MS) based analytical procedure after a QuEChERS extraction to determine the concentrations of these substances in the larvae. The GC-MS/MS method was used for the larval samples because it is generally more sensitive for analysis of (low-polarity) pyrethroids than LC-MS/MS (Murcia-Morales *et al.*, 2019; Kim *et al.*, 2022), especially in case of the comparatively high fat content of BSFL (Barragan-Fonseca *et al.*, 2017). Based on the analyzed concentrations, carryover, defined as the concentration in the larvae as a percentage of the concentration in the feed, was calculated. The procedures for the chemical analyses, as well as the analytical quality control results, are presented in detail in Appendix A.

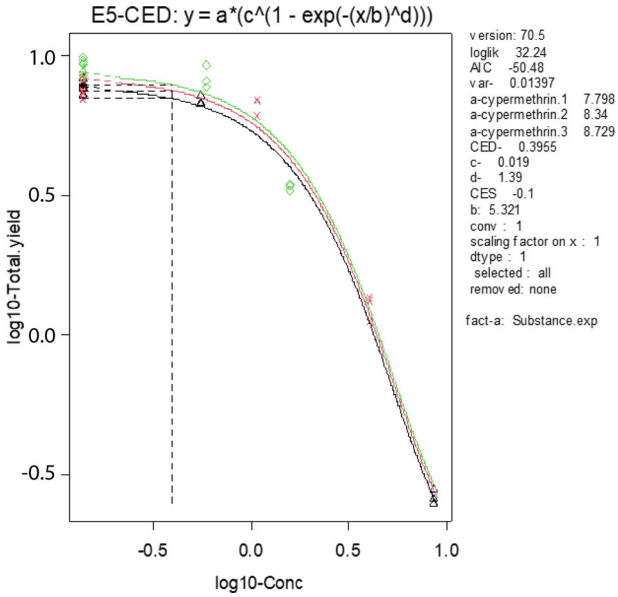

Fig. 1 Concentration/response curves for the effect of the concentration (mg/kg) of cypermethrin (CYP) on total black soldier fly larvae (BSFL) biomass yield (g) on log10-scale axes. The colors in the plot relate to the following subgroups (experiments): black/triangle (Experiment 1), red/cross (Experiment 2), green/diamond (Experiment 3).

Monitoring data

For this study, ForFarmers Corporate Services BV provided data on the presence of insecticides in feed materials, gathered via their yearly monitoring program of animal feed products during the 2020 and 2021 harvests. Samples were analyzed by EN-ISO17025:2017 accredited external laboratory Primoris (Belac Accreditation number: 057), using a multiresidue-method employing GC-MS/MS and LC-MS/MS. These monitoring data of insecticide residues in commercial animal feed are shown in Table C1 in Appendix C. In total 90 samples were analyzed to determine the presence and concentration of a wide variety of pesticides. The table only presents the 31 samples in which one or more of the pesticides used in this study were found; other substances and negative samples have been excluded from the presented data. Similar monitoring data were provided by Bestico B.V., as related to monitoring data for the feed ingredients which they used to produce their BSFL substrate in 2017−2022. These samples were analyzed by Eurofins in a manner similar as described for the data provided by ForFarmers, and again only data for tested substances are presented. The results of experimentally assayed concentrations of tested insecticidal substances in this study were compared against these monitoring data in terms of occurrence and concentrations.

Results and discussion

Quality control

The analyzed concentrations, and observed survival (%), biomass yield (g), and mean individual larval weight (mg) results are shown in Table B1 for all treatments. For each of the three experiments, no significant differences in the distribution of survival, yield, and individual larval weight between the three control treatments ($P > 0.05$) were observed. As such, the $n = 9$ control replicates included in each experiment were pooled for further statistical analysis. Quality control results of the analytical procedures are provided in Appendix A.

Cypermethrin (CYP)

The model with the best fit for the C/R relationship for CYP was the E5-CED model, with the experiment number (1, 2, and 3) as a covariate (Fig. 1). The AIC value of this model was 50.48. The software classified the yield

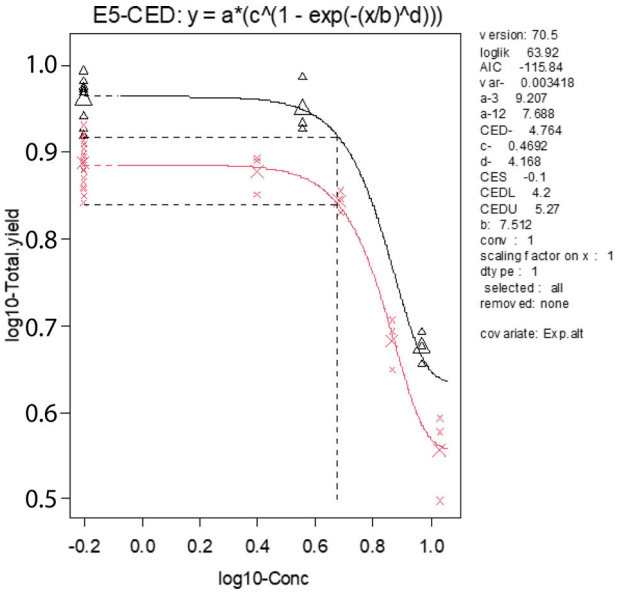

Fig. 2 Concentration/response curves for the effect of the concentration (mg/kg) of pirimiphos-methyl (PM) on the total black soldier fly larvae (BSFL) biomass yield (g) on log10-scale axes. The colors in the plot relate to the following subgroups (experiments): red/cross (Experiments 1 and 2), black/triangle (Experiment 3).

for the treatment with analyzed concentration 1.83 mg/kg in Experiment 1 as an outlier; this value was therefore removed from further analysis. The estimated CED for 10% yield, indicated by the dotted lines, is 0.40 mg/kg, with a 90% CI of 0.31−0.50 mg/kg. The estimated effect size for the concentration of the MRL of wheat (2.0 mg/kg) in terms of reduction in yield would be 59.2% (90% CI: 54.7%−62.7%). After correction for the volume of water added to the dry feed to obtain the exposure concentration (0.7 mg/kg), the estimated effect size at that concentration would be 20.5% yield (90% CI: 15.8%−25.0%). These findings indicate that the current MRL for CYP in wheat is insufficient to ensure optimal BSFL yields. Although the CYP concentrations found in the analyzed samples presented in Appendix C are mostly lower than 0.7 mg/kg, the highest concentrations (up to 0.56 mg/kg) may still cause significant yield loss.

Pirimiphos-methyl (PM)

The model with the best fit for the C/R relationship for PM was the same E5-CED model as for CYP, but with an alternative covariate: Experiments 1 and 2 were considered equivalent (Fig. 2; plotted as red line, crosses), and separate from Experiment 3 (plotted as black line, triangles). The AIC of this model was 115.84. The estimated CED for 10% yield, indicated by the dotted lines, is 4.76 mg/kg, with a 90% CI of 4.20–5.27 mg/kg, respectively. The MRL for PM in wheat is 5.0 mg/kg, which corresponds to an estimated effect size in terms of reduction in yield of 11.9% (8.1%−16.0%). After correction for the volume of water added to the dry feed to obtain the exposure concentration (1.75 mg/kg), the estimated effect size was 0.17% (0.05%−0.69%). Concentrations of PM in feed that do not exceed the MRL and, therefore, did not affect yield. The monitoring data shown in Tables C1 and C2 suggest that PM tends to be present in feed at substantially lower concentrations than tested in this study, implying a low chance of yield losses resulting from the presence of this substance in feeds at concentrations commonly found in commercial feed samples.

Piperonyl butoxide (PBO) synergism

The results for all treatments containing both CYP and PBO (PBO) at different ratios are shown in Fig. 3. CYP and PBO were tested at ratios of 1 : 1, 1 : 10, and 1 : 20 in Experiment 1, for CYP concentrations of 0.5 and 2.0 mg/kg. For both tested CYP concentrations at these different ratios relative to PBO, the variables yield and mean individual larval weight show a downward trend as the PBO concentration increased, while survival remained stable (Fig. 2). Based on these findings, it was decided to continue testing with a ratio of 1 : 20 in Experiments 2 and 3, in order to gather more data for a C/R curve.

The yield results for treatments in all three experiments containing CYP and PBO at a ratio of 1 : 20 were used for C/R in PROAST. First, the results were plotted by considering only the CYP concentration in these treatments, that is, taking the PBO ratio as a constant. The AIC of the E5-CED model with experiment number as a covariate (as used for CYP) was 36.42. However, the E3-CED model with the same covariate appeared to fit better (AIC of 40.2). As such, this E3-CED model was selected for further calculations and plotted (Fig. 4). The better fit of the E3 model compared to the E5 model in this case does not imply that the curve does not level off, but rather that the data collected for the higher assayed concentrations may not yet have resulted in the minimum value. In practical terms, since the focus of this research is on determining acceptable yield losses at lower concentrations, differences between the two models are negligible. The estimated CED for 10% yield is 0.22 mg/kg, with a 90% CI of 0.18−0.27 mg/kg. The estimated effect size in terms of reduction in yield corresponding to the

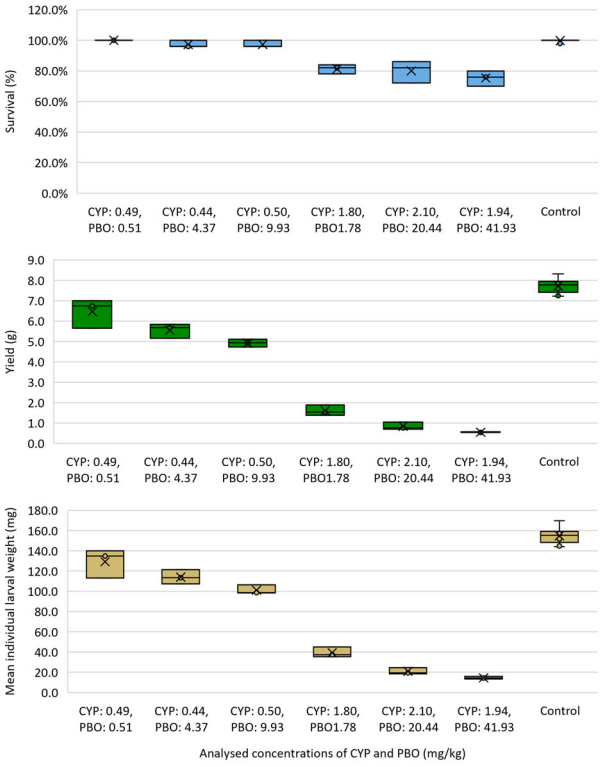

Fig. 3 Survival (%), yield (g), and mean individual larval weight (mg) in response to exposure to both cypermethrin (CYP) and piperonyl butoxide (PBO), for the concentrations analyzed (mg/kg). Box plots show median (-) and quartiles; X = arithmetic mean for $n = 3$ replicates per treatment, and $n = 9$ for the pooled control.

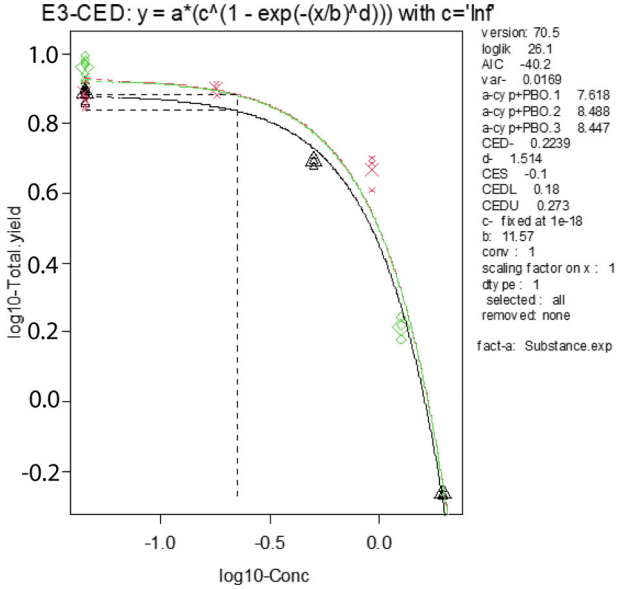

Fig. 4 Concentration/response curves for the effect of the concentration (mg/kg) of cypermethrin (CYP) in combination with piperonyl butoxide (PBO) on total black soldier fly larvae (BSFL) biomass yield (g) on log10-scale axes. The colors in the plot relate to the following subgroups (experiments): black/triangle (Experiment 1), red/cross (Experiment 2), green/diamond (Experiment 3).

aforementioned MRL in wheat of 2.0 mg/kg would be 94.0% (90% CI: 93.0%–94.7%). Corrected for the wet weight (0.7 mg/kg), the estimated effect size would be 44.5% (90% CI: 39.6%–48.6%).

Results of the modeling analysis on the relative potency of PBO when used in conjunction with CYP are shown in Fig. 5. Covariates for this model (AIC 78.06) were the substance and experiment number (a: CYP 1, 2, 3; CYP + PBO 1, 2, 3) and substance (CYP; CYP + PBO). The CED for CYP in this model was 0.59 mg/kg, with a 90% CI of 0.46−0.76 mg/kg. For CYP + PBO, the CED was 0.23 mg/kg, and the 90% CI of 0.18−0.29 mg/kg. The relative potency factor was 2.6 with 90% CI of 2.4−2.8.

Direct negative effects on BSFL yields were observed for CYP concentrations below the applicable MRL. Existing EC legal limits therefore appear not to be adequate to prevent yield loss and compromise larval health of mass-reared BSFL, as discussed in Cypermethrin (CYP) section. Since CYP is used as a plant protection product in conjunction with PBO, we would recommend using the CEDL-10 for CYP combined with PBO (BMDL of 0.18 mg/kg in wet feed, corresponding to 0.51 mg/kg in dry material), rather than the existing limit of 2.0 mg/kg, when using the feed material as insect substrate ingredient. Applying a lower maximum yield reduction (e.g., 5%) would of course reduce the threshold even further. Although less PBO relative to CYP is expected to be less toxic, caution is advised, especially if other insecticidal substances that may be synergized by PBO are also present in the feed.

PM was tested at a concentration of ∼10.0 mg/kg in conjunction with PBO, with ratios of 1 : 1 and 1 : 4, shown in Fig. 6. All observed variables showed significant differences with the controls ($P \leq 0.01$). Survival, yield, and mean individual larval weight decreased at increasing concentrations of PBO. The addition of PBO to the bioactivated organophosphate PM therefore seems to increase mortality and reduce yield.

Analogues and combined treatments

Pyrethroids DEL showed to be more toxic than CYP and PER, resulting in a mean yield of 5.9% of the

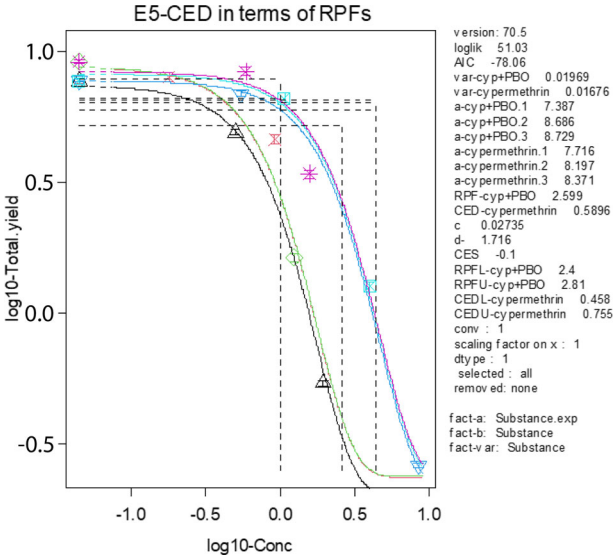

Fig. 5 Concentration/response curve for the relative potency factor (RPF) of the effect of the concentration (mg/kg) on total black soldier fly larvae (BSFL) biomass yield (g), for cypermethrin (CYP) combined with piperonyl butoxide (PBO) against CYP alone on log10-scale axes. The colors in the plot relate to the following subgroups (experiments): black/upward triangle (CYP + PBO, Experiment 1), red/cross (CYP + PBO, Experiment 2), green/diamond (CYP + PBO, Experiment 3); dark blue/downward triangle (CYP, Experiment 1), light blue/cross-square (CYP, Experiment 2), pink/cross-plus (CYP, Experiment 3).

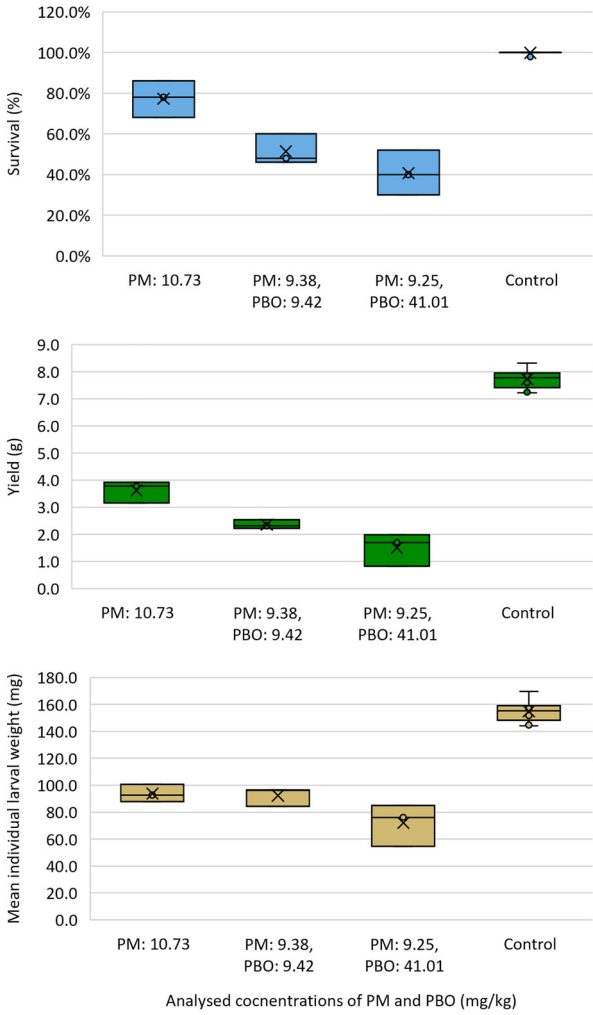

Fig. 6 Survival (%), yield (g), and mean individual larval weight (mg) in response to exposure to both pirimiphos-methyl (PM) and piperonyl butoxide (PBO), for the concentrations analyzed (mg/kg). Box plots show median (-) and quartiles; X = arithmetic mean for $n = 3$ replicates per treatment, and $n = 9$ for the pooled control.

controls, even at 0.53 mg/kg (Fig. 7). This severe reduction in yield suggests that the existing limit (1.0 mg/kg dry feed; 0.35 mg/kg exposure concentration in wet feed) is likely too high to prevent negative effects on insect performance. For insect rearing, we would therefore recommend using a threshold equal to the analytical limit of quantification of DEL, pending additional research on the effects of lower concentrations. The results for the treatment with all three pyrethroids combined were similar to those for DEL alone. At the lower tested concentration (~0.5 mg/kg), neither CYP nor PER had a significant effect on survival, yield, and mean individual larval weight ($P > 0.01$). The observed order of toxicity (DEL > CYP > PER) is in line with the consensus of their potency to insects in general (Bradbury & Coats, 1989). The higher toxicity of deltamethrin and cypermethrin compared to permethrin is likely attributable to the presence of the α-cyano group (Khambay & Jewess, 2005). Identifying the cause for the substantial difference between toxicity of cypermethrin and deltamethrin to BSFL is less straightforward. Several studies on other Diptera species appear to attribute such differences to development of resistance as a result of repeated or continued exposure to one or the other substance (Liu & Yue, 2000; Zhang *et al.*, 2007; Cetin *et al.*, 2010). This hypothesized cause would be in line with the higher incidence of cypermethrin than deltamethrin in the historically used substrate of the assayed population, as presented in Table C2. The results for the combined treatment are likely to be attributed to the comparatively high toxicity of DEL, which makes it difficult to draw conclusions on potential synergism among the three tested pyrethroids. More research, first employing lower concentrations of DEL in isolation, is needed before any joint effects can be tested.

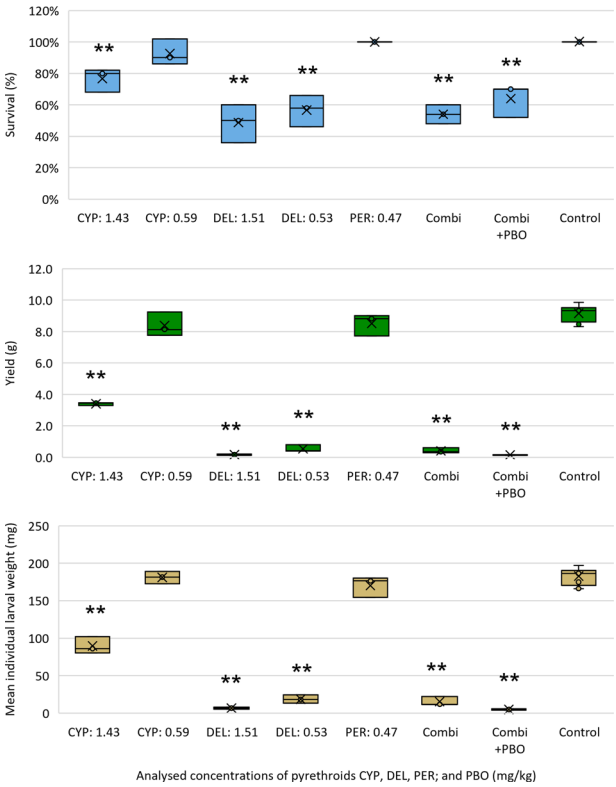

Fig. 7 Survival (%), yield (g), and mean individual larval weight (mg) in response to exposure to pyrethroids cypermethrin (CYP), deltamethrin (DEL), and permethrin (PER); all combined (combi), with or without the synergist piperonyl butoxide (PBO); for the concentrations analyzed (mg/kg). Box plots show median (-) and quartiles; X = arithmetic mean for $n = 3$ replicates per treatment, and $n = 9$ for the pooled control. Significance of differences between a treatment and the pooled control ($P < 0.01$) is denoted by **.

The addition of PBO to the three pyrethroids at the lower concentration caused a further reduction in total yield: it resulted in a yield value approximately equal to that of DEL at triple that concentration. Research on other species of Diptera (e.g., mosquitoes (family Culicidae) and housefly (*Musca domestica* L., Muscidae)) showed that PBO is an effective synergist for pyrethroids (Cakir *et al.*, 2008; Fakoorziba *et al.*, 2009; Darriet & Chandre, 2013; Koou *et al.*, 2014), but unfortunately few published data on *Hermetia illucens* specifically are currently available.

One of the objectives of this study was to determine to what extent the toxicity of an insecticidal substance could be extrapolated to other substances in the same class, or with a similar mode of action (MoA). The results showed clear differences between the three tested pyrethroids, suggesting that such an extrapolation has severe limitations. This implies a need to investigate the effects of each substance separately, which comes at higher costs. The virtually endless variety of possible compositions of insecticide "cocktails" (i.e., containing multiple substances at varying concentrations) that could be present in compound feed substrates complicate this matter further. The observed additive and synergistic effects resulting from exposure to multiple substances at low concentrations warrant more research. We recommend that a risk-based approach using monitoring data is employed in the prioritization of substances and combinations to study further. The first step to this approach involves an assessment of the relative risk of a hazard (i.e., an insecticidal substance) in relation to the intended feed source (i.e., the substrate), in terms of the probability of contamination and health effects for the insects and intended consumer (human/animal) of the insect (Focker *et al.*, 2022). Such insights could subsequently be used to develop a sampling and monitoring strategy (Van Asselt *et al.*, 2018; Van der Fels-Klerx *et al.*, 2018). For instance, a model developed by Wang *et al.* (2020) for aflatoxins and dioxins in dairy cow feed could be adapted to the risk of pesticides to reared insects.

From the monitoring data presented in Table C1, a total of 22 samples tested positive for PBO, of which 19 also contained CYP and/or DEL. Ratios of CYP to PBO in this dataset were slightly lower (1 : 5) than tested in this study (1 : 20), although comparatively higher ratios were reported for DEL (1 : 48). The highest concentration of DEL was 1.1 mg/kg, in a sample also containing PBO (4.2 mg/kg, ratio: 1 : 3.8). The results from this study suggest that concentrations of tested pyrethroids in commercial feed samples have the potential to cause significant reductions in BSFL yield.

Organophosphates At the lower concentrations, only CM resulted in a significant reduction in yield: for PM and MAL, this was not the case ($P > 0.01$; Fig. 8). At the higher tested concentrations, all three substances resulted in a significant reduction in yield ($P \leq 0.01$), but again the effect of CM was strongest. The effect of all three substances combined at lower concentrations was approximately equal to the average of each of the three single substances at the respective higher concentration in isolation, which suggests that the total combined effect was cumulative. The addition of PBO to the combined organophosphates resulted in a further substantial reduction in yield. This is interpreted as further evidence that PBO also increases mortality (and other adverse sublethal effects) of BSFL when exposed

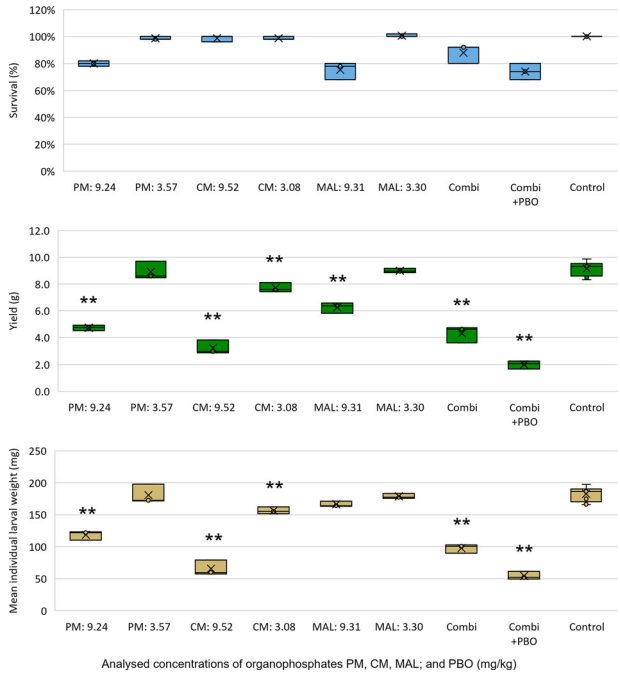

Fig. 8 Survival (%), yield (g), and mean individual larval weight (mg) in response to exposure to organophosphates pirimiphos-methyl (PM) chlorpyrifos-methyl (CM), and malathion (MAL); all combined (combi), with or without the synergist piperonyl butoxide (PBO); for the concentrations analyzed (mg/kg). Box plots show median (-) and quartiles; X = arithmetic mean for $n = 3$ replicates per treatment, and $n = 9$ for the pooled control. Significance of differences between a treatment and the pooled control ($P < 0.01$) is denoted by **.

to bioactivated organophosphates, as discussed in Piperonyl butoxide (PBO) synergism section.

CM and PER are no longer authorized for use in the EU (Regulations (EU) 2002/18 and Decision 2000/817/EC, respectively). Their legal limits are therefore set at the lower limit of analytical determination (0.01 mg/kg). Based on the relatively low reductions in yield at much higher concentrations (7.1% at 0.47 mg/kg and 15.8% at 3.08 mg/kg, respectively), these limits can be considered adequate to prevent reduction in BSFL yield for both these substances. For MAL, with a corrected MRL of 2.80 mg/kg, the statistically unaffected yields at 3.30 mg/kg also suggest that the current limit is adequate to prevent BSFL yield reduction. The concentrations found in commercial samples (presented in Appendix C) suggest that these tend to be relatively low compared to the concentrations tested in this study, which would cause less yield reduction. However, as highlighted in a previous exploratory study on the effects of insecticides on BSFL (Meijer *et al.*, 2021), an observed lack of direct effects on the tested larval stage does not preclude the possibility of larval exposure resulting in sublethal negative effects manifesting in later stages of the insect such as pupae and adults. We therefore stress caution in applying these findings in commercial facilities without validation of the absence of long-term effects and reiterate the need for more research into sublethal effects and chronic exposure to low concentrations.

Combined effect of CYP/PM/PBO The model substances CYP and PM were tested in conjunction with one another, and with PBO. Neither the treatment with, nor without PBO at lower concentrations showed a significant difference between the treatment and the controls. A different effect was observed at the higher concentrations, however. As discussed in Cypermethrin (CYP) section, the estimated effect at concentrations equal to the uncorrected MRLs for CYP (2.0 mg/kg) and PM (5.0 mg/kg) would be 59.2% and 11.9%. The substantially lower yield (80.6%) observed in the treatment combining these two substances suggests some degree of synergism between these pesticides of different classes, which is likely to be attributable to their different modes of action.

Individual larval weight Fig. 9 shows a box-plot of individual larval weights recorded in Experiment 3. The numerical data are shown in Table B2. For some treatments such as CYP at 1.43 mg/kg (82 ± 49 mg) and at 0.59 mg/kg (172 ± 38 mg), and pirimiphos-methyl at 9.24 mg/kg (122 ± 41 mg/kg) the standard deviation was substantially higher than for the pooled controls (184 ± 24 mg). Although this observation is based on limited data, it may have commercial consequences: exposure to insecticide residues in the substrate may not only result in lower yields, but also in lower product homogeneity—and thus lower quality.

Concentrations in larvae

Table 2 presents the analyzed concentrations of tested substances in the feed compared to the concentrations in the larvae. For most substances, the carryover (defined as the concentration in the larvae as a percentage of the concentration in the feed) was low (≤ 10%), and bioaccumulation (transfer > 100%) did not occur for any treatment. This is in line with previous findings for BSFL on absence of bioaccumulation (Meijer *et al.*, 2021). For all tested organophosphates, the analyzed concentration in the larval samples was < 5% of the concentration in the feed. This was slightly higher for the pyrethroids: the highest larval concentration was observed for the larvae exposed to DEL (1.14 mg/kg). A potential link

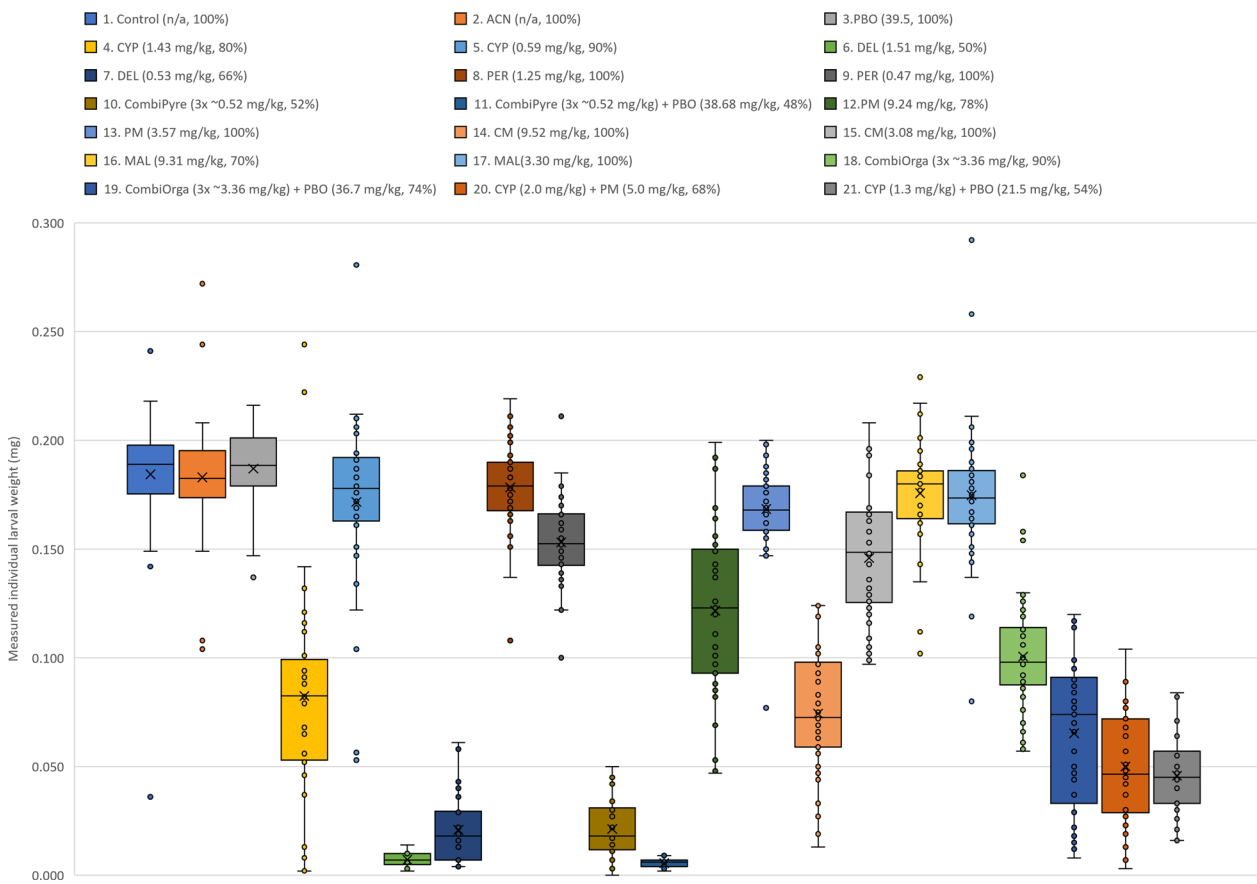

Fig. 9 Results for treatments tested in Experiment 3, in terms of actual individual black soldier fly larval weight (mg) in response to exposure to tested insecticides. Pyrethroid (Pyre) substances tested were cypermethrin (CYP), deltamethrin (DEL), Permethrin (PER); organophosphates (Orga) substances were pirimiphos-methyl (PM), chlorpyrifos-methyl (CM), malathion (MAL); synergist was piperonyl butoxide (PBO). Median (-) and quartiles. Analyzed concentration and survival in replicate indicated in brackets. X = arithmetic mean.

between larval concentration and performance (yield/larval weight) is, however, not clear: the larval concentrations of DEL in the combined treatment with CYP and PER were not elevated, despite drastic reductions in performance in response to those treatments. However, the addition of PBO to the treatment with all three pyrethroids clearly had an effect on transfer: this was more than twice as high for all three substances. This observation is in line with the intended effect of PBO to inhibit activity of P450- and esterase enzymes, thereby blocking metabolic conversion (Snoeck *et al.*, 2017). More research is needed to determine which factors mediate transfer of insecticides to BSFL biomass and the role of PBO therein.

In EU legislation, MRLs have been set for all tested substances both in wheat and insects (terrestrial invertebrate animals)—with the exception of permethrin in insects. The MRLs for insects have all been set at the substance-specific default, denoted by an asterisk, which is 0.01× mg/kg for PM and CM; 0.02× mg/kg for DEL and MAL; and 0.05× mg/kg for CYP. Assuming a worst-case scenario, that is the maximum allowed concentration in the wheat substrate (corrected for the 35% dilution by addition of water), and the highest observed transfer rate, then there is a plausible risk of noncompliance for CYP and DEL. The highest transfer rates observed for these substances were 30.8% (in combination with PER and DEL) and 75.5% (in isolation), respectively. The treatments in which those transfer rates were observed also caused exceptionally high reductions in yields, which makes commercial use of insects exposed to such concentrations unlikely. As hypothesized above, lower concentrations, resulting in lower reductions in yield, are likely to be associated with lower transfer rates.

Table 2 Concentrations of spiked substances in BSF larval samples in Experiment 3, compared to analyzed concentrations of respective substances in provided feed, and percentage of carryover (defined as the concentration in the feed as a percentage of the concentration in the larvae), for the substances piperonyl butoxide (PBO), cypermethrin (CYP), deltamethrin (DEL), permethrin (PER), pirimiphos-methyl (PM), chlorpyrifos-methyl (CM), and malathion (MAL).

Substance	Analyzed concentration feed (mg/kg)	Analyzed concentration larvae (mg/kg)	Percentage carry over (%)
PBO	39.52	0.29	0.7%
CYP	1.43	0.25	17.5%
CYP	0.59	0.11	18.6%
DEL	1.51	1.14	75.5%
DEL	0.53	0.16	30.2%
PER	0.47	0.07	14.9%
CYP	0.47	0.06	12.8%
DEL	0.48	0.05	10.4%
PER	0.48	0.04	8.3%
CYP	0.52	0.16	30.8%
DEL	0.57	0.15	26.3%
PER	0.53	0.14	26.4%
PBO	38.68	7.42	19.2%
PM	9.24	0.16	1.7%
PM	3.57	0.02	0.6%
CM	9.52	0.05	0.5%
CM	3.08	0.03	1.0%
MAL	9.31	0.05	0.5%
MAL	3.30	0.03	0.9%
PM	3.68	0.02	0.5%
CM	3.01	0.01	0.3%
MAL	3.03	0.00	0.0%
PM	3.10	0.06	1.9%
CM	3.01	0.04	1.3%
MAL	2.92	0.00	0.0%
PBO	36.68	0.26	0.7%
CYP	1.95	0.19	9.7%
PM	5.25	0.10	1.9%
CYP	1.25	0.13	10.4%
PBO	21.49	0.54	2.5%

Nevertheless, this finding highlights the need for reduced concentrations of CYP and DEL in BSFL substrate not just to avoid reduced yields, but also to ensure compliance with existing MRLs in insect biomass. Transfer rates for the tested organophosphates PM, CM, and MAL were much lower (< 2%). Since the MRL of CM in wheat is also at a default 0.01× mg/kg, there is no anticipated risk of noncompliance, nor reduced yields, due to exposure to that concentration. However, for PM and MAL, the MRLs in wheat are higher than for the pyrethroids, at 5.0 and 8.0 mg/kg, respectively, which makes noncompliance in a worst-case scenario a theoretical possibility.

Conclusion and recommendations

For CYP and DEL, the tested concentrations that resulted in a significant reduction in BSF larval biomass yield in the present study match those found in feedstuff currently available in commercial practice. Furthermore, observed transfer rates from substrate to insect biomass coupled with the low default MRLs of these substances in insects introduce a risk of noncompliance in a worst-case scenario. As such, it is advisable that the insect rearing industry analyses incoming feed materials for the levels of CYP, DEL, and PBO specifically, since the currently applicable MRLs for these substances are not adequate to prevent reductions in BSFL yield. For CYP in the presence of PBO, a lower limit for a critical effect dose (CED) of 10% would be 0.18 mg/kg. A specific limit cannot be provided for DEL, given its severe toxicity. Absence of DEL in feed materials (< LOQ) used for BSFL rearing is therefore recommended, pending further research. The results for the tested organophosphates (PM, CM, MAL) and PER did not lead to recommendation of lower limits than those currently laid down in EU legislation. The results of this study suggest that extrapolation of effects of substances with the same mode of action is not warranted, which implies a need to assess each substance separately. We anticipate that some other commonly used insecticides, which have thus far not been tested, may also exert negative effects on BSFL health. For EU policymakers, we would advise considering adopting insect species-specific limits in legislation (e.g., Directive 2002/32/EC), or via adoption of harmonized guidance levels through other means than legislation. More research is needed to answer several questions raised based on the current results, but that should not need to delay preparation of additional legislative (for policymakers) or contractual (for industry) thresholds. Specific questions for future research are to what extent insecticide residues can cause sublethal effects, what the effects are on other commonly reared insect species, which (toxic) metabolites may be formed during exposure, what the effects are of multiple insecticides at lower concentrations, and which mechanisms or measures might be employed to reduce negative effects. Additional research is

especially pertinent on the effects and synergism of combinations of insecticide residues in various feed materials, since insect substrates are often mixed consisting of a variety of ingredients from different suppliers or batches or suppliers.

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Disclosure

The Ministry had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The commercial entities within the project consortium were Ynsect NL Nutrition & Health B.V.; Protix Biosystems; Bestico B.V.; and ForFarmers. With the exception of Bestico B.V., the commercial entities in the consortium had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Bestico B.V. uses black soldier fly larvae to convert biomass into protein and oil for feeding purposes, and fertilizers. Bestico B.V. provided in-kind contribution to the project primarily in the form of labor costs for author L.Z., who assisted in the execution of experiments, which were performed at the premises of that company under supervision of the corresponding author. Bestico B.V. otherwise had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix A Method for chemical analyses and quality control (QC) results.

Table A1 Used insecticidal substances, class and suppliers.

Table A2 LC-MS/MS conditions.

Table A3 GC-MS/MS conditions, MS/MS transitions.

Table A4 Results of the analyses to verify homogeneous distribution of spiked substances cypermethrin (CYP), pirimiphos-methyl (PM), and piperonyl butoxide (PBO) in black soldier fly larvae diet.

Table A5 Recovery percentages of analytical series to verify concentration of spiked substances in black soldier fly larvae diet.

Table A6 Recovery percentages of analytical series to verify concentration of spiked substances in blank black soldier fly larvae.

Appendix B Overview of experimental results.

Table B1 Overview of results from three executed experiments.

Table B2 Overview of individual larval weight (mg), based on individually weighed larvae in Exp. 3.

Appendix C Monitoring data for insect feeds.

Table C1 Monitoring data from project partner ForFarmers for commercial feed ingredients, including product type, country of origin, and sample date.

Table C2 Monitoring data from project partner Bestico B.V. for commercial feed ingredients used in substrate flor black soldier fly larvae (BSFL), including product type and sample date.

Appendix D Small-scale neonate larvae experiment.

Table D1 Results of small-scale experiment with insecticides using neonate larvae (D1-7).