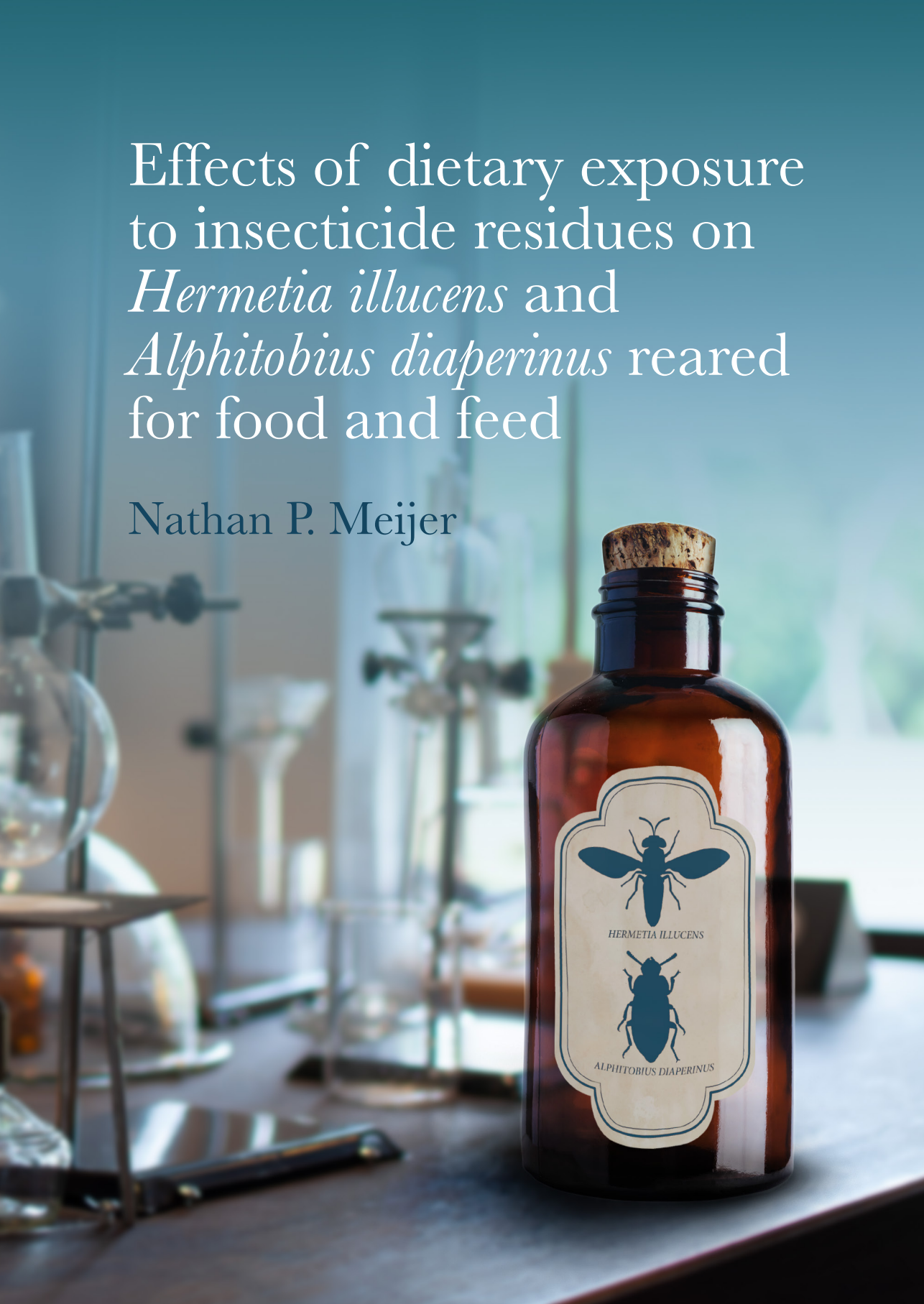
Effects of dietary exposure to insecticide residues on *Hermetia illucens* and *Alphitobius diaperinus* reared for food and feed

Nathan P. Meijer



Propositions

1. Existing insecticide residue limits for feed materials as laid down in European Union legislation are inadequate to safeguard survival and growth of reared insects.
(this thesis)

2. The commonly occurring presence of cocktails of insecticide residues in fruit and vegetable waste makes this substrate unsuitable for rearing insects.
(this thesis)

3. To implement scientific findings in legislation, direct knowledge exchange between scientists and legislators is imperative.

4. Scientists underestimate the impact of visual storytelling to amplify the societal impact of their scientific results.

5. Replacing wild-caught fish-meal in feed for aquaculture animals with alternative proteins is a key step towards reducing the ecological footprint of the aquaculture industry.

6. The effects of climate change on insect diversity and population numbers will be catastrophic for agricultural production.

Propositions belonging to the thesis, entitled

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Nathan Meijer
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Effects of dietary exposure to insecticide residues on *Hermetia illucens* and *Alphitobius diaperinus* reared for food and feed

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Chapter 1

General introduction

(Hermetia illucens and Alphitobius diaperinus in chemical laboratory. Models designed and produced by Assemblishop.nl, produced for the H2020 project SUSINCHAIN (SUSINCHAIN.EU).Photo by author.)

Introduction

Insects reared specifically for purposes of food and animal feed are considered to be a promising new alternative that can contribute to the increasing global demand for sustainable proteins (Guiné et al., 2021; Van Huis & Oonincx, 2017; Van Huis et al., 2013). Insects are a major component of the natural diet of 'conventional' livestock and the use of insects as feed in commercial poultry and pig farming systems could improve welfare aspects (Dörper et al., 2021; Sogari et al., 2022; Szendrő et al., 2020; Veldkamp & Vernooij, 2021). Furthermore, various insect species that are collected from the wild have a long history of human consumption in Africa and South-East Asia (Chen et al., 1998; Van Huis, 2003), and the number of small-scale rearing facilities in those countries have been increasing steadily (Durst & Hanboonsong, 2015; Raheem et al., 2019). Due to the limited history of use of insects as food and feed in the European Union (EU), a precautionary approach is employed and certain safety assurances must be met before insect-based products can legally be marketed for feed or food in Europe (Skotnicka et al., 2021; Svanberg & Berggren, 2021). An increasing amount of research has been performed on the safety of insects for human food and animal feed in the past decade and the first insect products have now been approved for food and feed purposes. Nevertheless, many safety aspects related to use of insects as food or feed are at this time still uncertain (EFSA, 2015; Van der Fels-Klerx et al., 2018). For instance, the heavy metals cadmium and arsenic have been shown to bio-accumulate to varying degrees in, respectively, black soldier fly larvae (BSFL, *Hermetia illucens* (L.); Diptera: Stratiomyidae) and yellow mealworm (YMW, *Tenebrio molitor* (L.); Coleoptera: Tenebrionidae), but the specific factors that influence the final concentrations in the insect body are unclear (Biancarosa et al., 2018; Diener et al., 2015; Purschke et al., 2017; Van der Fels-Klerx et al., 2016). In contrast, certain toxins produced by fungi (named 'mycotoxins') such as aflatoxin B1 appear to be absent in the larval biomass, and may even be metabolically broken down – although again the exact mechanisms are as of yet uncertain (Bosch et al., 2017; Camenzuli et al., 2018; Meijer et al., 2019). Other categories of chemical contaminants that have been the subject of research in relation to reared insects include environmental contaminants such as dioxins and (dioxin-like) polychlorinated biphenyls (PCBs) (Pajurek et al., 2023; Van der Fels-Klerx et al., 2020) and veterinary drugs (Hoek-Van den Hil et al., 2022), among others.

One particular category of hazards that has received comparatively limited attention until now are insecticides, which may be present in feed materials as residues from agricultural use. Insecticide residues in the substrate on which insects are reared may affect their growth and survival, thereby impacting the health of insects and the profitability of the insect industry. In addition, there is a potential risk of bioaccumulation of contaminants in the insects from the substrate on which they feed (EFSA, 2015). If such accumulation occurs, the concentration of insecticides in the insect products will become higher than in the feed substrate. Insecticide concentrations

in the final insect product that are too high may no longer be compliant with certain (legal) limits that are applicable to insects marketed as food or feed and may present a safety risk. 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), but these studies mainly investigated insect products that were offered for sale to consumers. A plethora of published research is available on the effects of synthetic insecticides on beneficial insects (Calvo-Agudo et al., 2022; Devine & Furlong, 2007; Siviter & Muth, 2020) as well as on insect pest and vector control (López et al., 2005; Stejskal et al., 2021; Van den Berg, 2009). For instance, several mealworm species that are now being reared for food and feed are traditionally considered pests in poultry farms and in stored grains, and literature on the effects of insecticides on such species for pest control is therefore abundant (Aribi et al., 2006; Kaufman et al., 2008; Renault & Colinet, 2021; Zafeiriadis et al., 2021) – but it is unclear to what extent those findings can be extrapolated to the conditions in commercial mass-rearing of edible insects. As far as I am aware, experimental research focusing specifically on the effects of insecticide residues on insects reared for food and feed purposes is limited to Purschke et al. (2017), who experimentally exposed BSFL to chlorpyrifos and chlorpyrifos-methyl, and pirimiphos-methyl, Tomberlin et al. (2002), who tested the effects of cyromazine, pyriproxifen, λ-cyhalothrin and permethrin on BSF larvae and adults, and Dreassi et al. (2020) who tested the effects and bioaccumulation of deltamethrin, tebuconazole and chlormequat chloride by YMW. The substance chlorpyrifos was also found in a sample of housefly (*Musca domestica* (L.), Diptera: Muscidae) in a study by Charlton et al. (2015), but they focused on testing a variety of naturally contaminated substrates rather than assaying specific substances. Experiments of Houbraken et al. (2016) included only a single insecticide (bifenthrin). The focus of their study was primarily on fungicides, as was the case for a study on the bioaccumulation of the fungicide metalaxyl in YMW by Gao et al. (2014), and of a study of Lalander et al. (2016) (azoxystrobin and propiconazole).

Legislation

In the EU, only specified insect species may legally be used for food or feed purposes, as listed in Table 1. At this time, a total of eight insect species are permitted to be used as 'processed animal protein' (PAP) in feed for aquaculture animals, and pigs and poultry (Regulation (EC) No 142/2011). If insects are intended to be marketed as food for human consumption, the producer must first apply for authorization from the European Commission, which involves evaluation of a detailed safety dossier by the European Food Safety Authority (EFSA) (Regulation (EU) 2015/2283 and 2017/2470). Of these listed species, BSFL and lesser mealworm (LMW, *Alphitobius diaperinus* (Panzer); Coleoptera: Tenebrionidae) were the focus of the research performed for this thesis. BSFL are capable of feeding on a large variety of organic materials, which makes them very suitable for waste management, as well as for the production of feed (Abd El-Hack et al., 2020; Barragan-Fonseca et al., 2017). The larval development from neonate until

pupa takes approximately 3 weeks (Wang & Shelomi, 2017), and high crude protein and lipid contents make them especially suitable for inclusion in feed formulations (Alagappan et al., 2022). LMW are traditionally considered pests in poultry farms (Rumbos et al., 2019), although consideration of their use as a feed source is not new (Despins & Axtell, 1994, 1995). LWM are smaller in size than other tenebrionid larvae such as YMW and 'superworms' (*Zophobas morio* (Fabricius); Coleoptera: Tenebrionidae). They also have a faster developmental cycle, higher reproduction capacity and softer exoskeleton (Kurečka et al., 2021).

Table 1: List of insect species that are permitted to be used for feed or food purposes according to European Union legislation (Compiled on 27 June 2023 from Regulation (EU) 2017/2470 and Regulation (EC) No 142/2011).

Common name	**Scientific name**	**Order: Family**	**Author citation**	**Feed**	**Food**
Black soldier fly	*Hermetia illucens*	Diptera: Stratiomyidae	Linnaeus, 1758	X	
Common housefly	*Musca domestica*	Diptera: Muscidae	Linnaeus, 1758	X	
Yellow mealworm	*Tenebrio molitor*	Coleoptera: Tenebrionidae	Linnaeus, 1758	X	X
Lesser mealworm	*Alphitobius diaperinus*	Coleoptera: Tenebrionidae	Panzer, 1797	X	X
House cricket	*Acheta domesticus*	Orthoptera: Gryllidae	Linnaeus, 1758	X	X
Banded cricket	*Gryllodes sigillatus*	Orthoptera: Gryllidae	Walker, F., 1869	X	
Field cricket	*Gryllus assimilis*	Orthoptera: Gryllidae	Fabricius, 1775	X	
Migratory locust	*Locusta migratoria*	Orthoptera: Acrididae	Linnaeus, 1758		X
Silkworm	*Bombyx mori*	Lepidoptera: Bombycidae	Linnaeus, 1758	X	

In the EU, insects reared for food or feed purposes are classified as 'farmed animals' (Regulation (EC) No 1069/2009, Article 3.6). As a consequence of this classification, specific rules apply to the materials that may be used to rear insects on, as well as on how insects may be fed to other animals (if used as feed). In principle, the substrates on which insects may be reared are largely limited to materials of vegetable origin. As discussed above, if insects are intended to be used as food for human consumers, a company must first have a detailed 'novel food' dossier on (amongst others) the safety of the product evaluated by EFSA (Regulation (EU) 2015/2283). Such authorizations are, in principle, product-, rather than company- or species-specific. An applicant can flag proprietary scientific evidence and scientific data in the dossier to be kept confidential for a period of five years. This effectively restricts the authorization to the applying company, although it does not preclude subsequent applicants to obtain authorization for that product, if submitted without reference to the mentioned proprietary evidence and data.

Pesticides, or 'plant protection products', may only be used if authorized, and specific legal limits apply. Several factors are taken into account for the authorisation of active substances used in insecticide formulations, but the general principles are that they should be applied in accordance with good agricultural practice (GAP) and that they may not have any harmful effects on human or animal health, and no unacceptable effect on the environment (Regulation (EC) No 1107/2009, Article 4.2). One of the specific requirements for the authorisation of pesticides is that the ecotoxicological risk is at an 'acceptable' level, which includes consideration of adverse effects on non-target organisms and the degree of exposure under 'realistic proposed conditions' (Regulation (EC) No 1107/2009, Annex II, section 3.8). Specifically, a compound is approved only if, 'following an appropriate risk assessment', its use under the proposed conditions results in negligible exposure of honeybees, causing no "unacceptable acute or chronic effects on colony survival and development" (section 3.8.3 of Regulation (EC) No 1107/2009). As such, most research on the ecotoxicity of pesticides has been performed on honeybees (see e.g., Sanchez-Bayo and Goka (2014)). However, it is unknown if available toxicological data on honeybees can be extrapolated to those insect species currently being mass-reared for food and feed. Due to a lack of published data, it is unclear if insecticide residues in feed materials may affect the health of insects reared for food and feed, and if the reared larvae to be used for feed and food contain increased levels of the insecticides as a result of accumulation.

In the EU, legal limits (maximum residue limits, MRLs) for the presence of insecticides in feed and food materials/products have been set in Regulation (EC) No 396/2005 and, to some extent, Directive 2002/32/EC. The latter directive is specific to feed products and lists MRLs for a variety of contaminants, but the pesticides listed in the directive are limited to ten insecticidal organochlorine compounds, such as DDT; it is therefore limited in scope. Conversely, Regulation (EC) No 396/2005 is applicable to both food and feed products and is, therefore, the framework legal instrument for pesticide MRLs. These MRLs are established for specific pesticide/commodity combinations and may therefore differ between different products. Concentrations of insecticide residues in food and feed are set at the lowest achievable level ('ALARA' principle: 'as low as reasonably achievable) consistent with GAP and the lowest consumer exposure necessary to protect vulnerable consumers (Regulation (EC) No 396/2005, Article 3.2d). In case of a pesticide that is not permitted to be used on a certain commodity, the MRL is set at the lowest limit of analytical determination, or a default of 0.01* mg/kg for those products for which there is no specific limit listed in Annex V of Regulation (EC) No 396/2005 (Article 18.1(b)). Certain exceptions to the default limit of 0.01* mg/kg may apply in case of difficult matrices (higher), or 'very low toxicological reference value' (DG SANTE, 2023). The pesticide MRLs listed for the commodity 'terrestrial invertebrate animals' (code number 1060000) are applicable to insect products if marketed for food or feed. Therefore, if pesticides are transferred (or bioaccumulate) from substrate to the insect biomass, the concentration in the insects must meet this

MRL for 'terrestrial invertebrate animals' in order to be compliant with the limits laid down in Regulation (EC) No 396/2005.

Insecticide chemical class and mode of action

A large variety of insecticides, in different chemical classes, is sold and used in the EU. Regulation (EU) 2017/269 provides an overview of these classes for statistical purposes. Eurostat (2023) reports that, of the insecticides that were classified, those based on pyrethroids (3.7 %), organophosphates (2.1 %), and carbamates and oxime-carbamates (0.8 %) saw the highest sales figures in 2021. These types of substances, as well as certain other classes of insecticides, employ a mode of action (MoA) that specifically interacts with insect nerve and muscle targets (Rajashekar & Shivanandappa, 2017). Other insecticide MoAs include mechanisms that affect growth, reproduction, and/or respiration. An overview and classification of the MoAs of most insecticides is provided by the Insecticide Resistance Action Committee (IRAC) (IRAC, n.d.).

A visual overview of the general structural formulas of selected examples of pyrethroid (A, permethrin and cypermethrin), organophosphate (B, pirimiphos-methyl), and carbamate (C, propoxur) insecticides is provided in Figure 1. Pyrethroids are composed of a large variety of synthetic analogues of natural pyrethrins from pyrethrum flowers (*Tanacetum* [formerly Chrysanthemum] *cinerariifolium* (Trevir.) Sch. Bip. (Asteraceae)) (Casida, 1980; Jeran et al., 2021; Katsuda, 1999). They are generally classified into two groups, that are associated with the absence (Type I, e.g. permethrin) or presence (Type II, e.g. cypermethrin) of an alpha-cyano group. The presence of this group appears to enhance insecticidal activity, although this differentiation appears to be simplified and does not fully explain differences in toxicity between pyrethroids (Khambay & Jewess, 2004; Kobayashi et al., 1989). Pyrethroids interact with the voltage-gated sodium channel, thereby disrupting nerve function and causing 'knockdown' and eventual death (Dong et al., 2014; Field et al., 2017). Organophosphates are characterized by the presence of phosphorus double bonded to oxygen and three other groups. Most insecticides are phosphorothioates, which contain a double bonded sulfur atom, replacing the oxygen atom, which is inherently more stable (WHO, 1986b). However, this P=S bond requires in vivo oxidative activation into the active phosphate analogue (P=O, 'oxon') form (Vale, 2015; Van Dyk & Pletschke, 2011). Carbamates are characterized by a carbonyl group (C=O) bonded with alkoxyl (OR) and amino (RNR) groups (Matošević & Bosak, 2020). In contrast to organophosphates, carbamates do not require bio-activation (WHO, 1986a). Both organophosphorus and carbamate insecticides target acetylcholinesterase (AChE), an enzyme that is needed for neurotransmission. Inactivation of this enzyme by such insecticides causes continuous stimulation of muscles and nerves, resulting in exhaustion and eventual death (Fukuto, 1990; WHO, 1986b). Both organophosphorus and carbamate insecticides are highly toxic to humans (Eddleston, 2020), while pyrethroids are much more specific to insects – although there have been reports of

(occupational) intoxication (Kaneko, 2011; Ramchandra et al., 2019). Although pyrethroids are highly toxic to aquatic wildlife under laboratory conditions, the ecotoxicology in field conditions appears to be less drastic due to relatively rapid degradation of the compound (Maund et al., 2012).

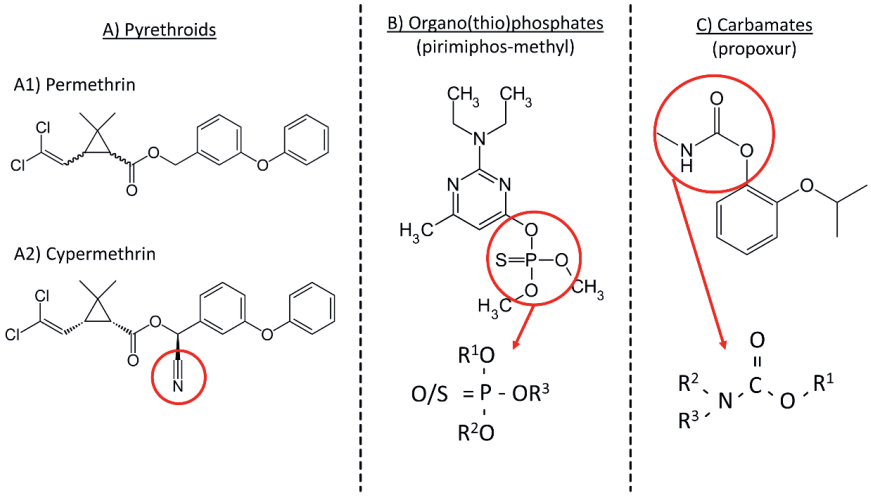

Figure 1: Structural formulas of examples of pyrethroids (A) [A1) permethrin; A2) cypermethrin], organothiophosphates (B), and carbamates (C). The red circles highlightcircles certain characteristics of each group: A) the alpha-cyano group that differentiates group I and II pyrethroids; B) the O=P(OR)₃ group in organophosphates and organo(thio)phosphates (P=S); and the carbamate ester functional group (R₂NC(O)OR).

The effects of harmful substances (e.g., insecticides) on insects may be modulated by several resistance mechanisms, including behavioural, penetration, target-site, and metabolic resistance (Siddiqui et al., 2023). Selected resistance mechanisms are shown graphically in Figure 2. The mechanism of metabolic resistance "often results from the overproduction of 'detoxification enzymes' that can metabolize xenobiotics" (Després et al., 2007). An overview of the main pathways of metabolic resistance to insecticides was given by Panini et al. (2016) who summarised these as follows: "detoxification can be divided into phase I (primary) processes, consisting of hydrolysis or oxidation, and phase II (secondary) processes, consisting of conjugation of phase I products with endogenous compounds". One of the most important enzyme families for insecticide detoxification is the group of Cytochrome P450 monooxygenases (P450s). The efficacy of metabolic resistance mechanisms may be up- and down-regulated by certain substances. For example, piperonyl butoxide (PBO) inhibits P450 enzymes (Després et al., 2007). PBO thus acts as a synergist to the insecticidal class of pyrethroids by preventing detoxification, which is why they are often used combined in commercial formulations (Davies et al., 2007). Target-site resistance results from a genetic mutation that affects the molecular site that is targeted by an insecticide, for instance, due to point mutations in a certain receptor or enzyme that prevent binding with an insecticide – thereby reducing the insect's sensitivity (Davies et al., 2008; Feyereisen, 1995). Multiple mechanisms may play a role: resistance to the aforementioned

pyrethroids can involve metabolic (CYP6D1) (Gao et al., 2012; Seifert & Scott, 2002) as well as target-site (knockdown resistance: kdr) (Donnelly et al., 2009) mechanisms, for instance. Furthermore, certain mechanisms can induce cross-resistance to multiple chemical classes: for example, resistance to the organochlorine insecticide DDT has been linked to pyrethroid resistance (Chadwick & Bowron, 1977; Prasittisuk & Busvine, 1977).

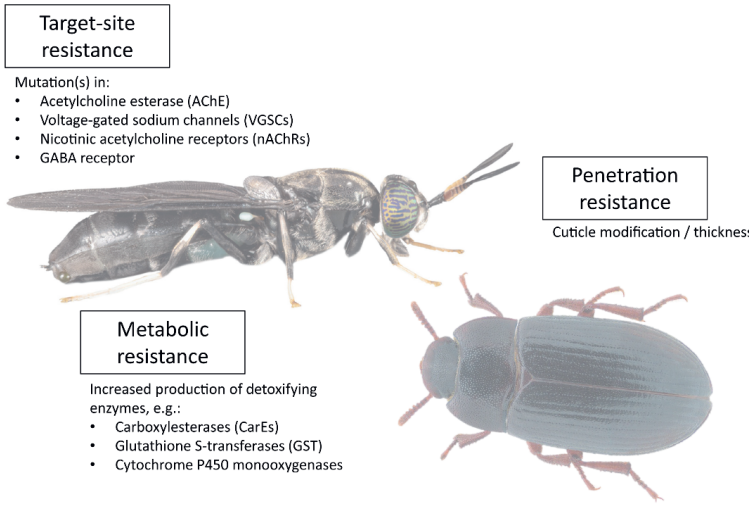

Figure 2: Overview of selected resistance mechanisms: target-site, metabolic, and penetration resistance. Figure based on Siddiqui et al. (2023). Photo of Hermetia illucens fly (top picture) by Hans Smid (www.bugsinspace.nl). Photo of Alphitobius diaperinus beetle (bottom picture) by Udo Schmidt (https://www.flickr.com/photos/coleoptera-us/32514044020/) (license: CC BY-SA 2.0).

The assessment of the toxic effects of insecticide residues on reared insects is complicated by the potential of 'cocktail' and sub-lethal effects. Firstly, a multitude or cocktail of insecticide residues may be present in the raw materials and compound feeds used for insect rearing, because rotating, alternating, or combining insecticides with different MoAs are proposed as methods to prevent or mitigate the development of insecticide (cross-)resistance (Simon-Delso et al., 2015; Sparks & Nauen, 2015). Furthermore, insect substrates are generally 'compound' feeds consisting of different (waste) materials, or sourced from different suppliers or production batches (Fuso et al., 2021; Mancini et al., 2019; Riekkinen et al., 2022; Yandi et al., 2023). Different insecticidal substances may interact with one another: additively or synergistically (i.e., amplification), or antagonistically (i.e., attenuation) (Sun & Johnson, 1960; Syberg et al., 2008). Secondly, the larval stage of reared insects is generally used for production of food or feed, however, in insect mass-production all life stages need to be maintained to allow reproduction for subsequent production batches. Other stages in the insect lifecycle than the larval stage may be more or less affected by insecticidal exposure, or performance may be affected by exposure in a previous stage (Arthur et al., 2018; Palmquist et al., 2008). Furthermore, certain substances may have effects at sub-lethal

exposure levels: the insect is not killed, but performance characteristics such as oviposition and pupation could be negatively affected after larval exposure (Desneux et al., 2007; Guedes et al., 2011). Both of these types of sub-lethal effects could result in decreased reproduction levels, or subsequent generations could be negatively affected.

Objectives

The primary objective of this thesis was to assess the effects of insecticide residues present in the feed substrate on BSFL and LMW performance and bioaccumulation. This primary objective was split into two sub-objectives: firstly, to assay the effects of selected insecticides on these two insect species, primarily in terms of survival and growth (yield). The second sub-objective was to determine transfer or accumulation of tested insecticidal substances from the substrate to the insect biomass, to be used for food or feed purposes. Specific attention was paid to the selection of insecticides to be investigated such that insecticides with various MoAs and those commonly found in organic residues were included. After initial toxicity assessment of a variety of substances for these two species, subsequent study designs focused on determining the effects of exposure to multiple combined insecticides (for BSFL); and on assaying sub-lethal effects on multiple generations (for LMW).

Outline of this thesis

This thesis consists of five interrelated chapters. Chapter 2-5 each report on an experimental study: two studies on BSFL, and two on LMW. Results from all four experimental studies are synthesized and discussed in Chapter 6. The structure and interconnections of these studies are outlined in Figure 3.

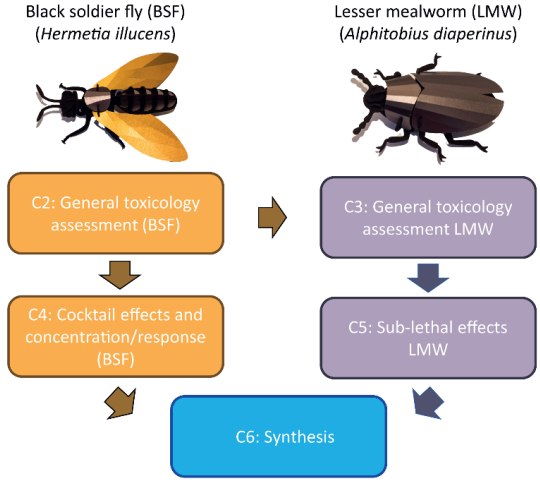

Figure 3: Graphical illustration of the outline of this thesis, showing the chapters on black soldier fly (BSF, Hermetia illucens) on the left (C2, 4); and the chapters on lesser mealworm (LMW, Alphitobius diaperinus) on the right (C3, 5); followed by a synthesis on all experimental results (C6). Images of models of Hermetia illucens and Alphitobius diaperinus by Joop Bource (Assemblishop.nl), produced for the H2020 project SUSINCHAIN (SUSINCHAIN.EU).

Chapter 2 describes the effects of insecticides with various MoAs on BSFL. The first aim of this study was to assess whether insecticidal substances commonly found in animal feed, at known concentrations equal to or below the applicable legal limit, could affect the survival and/or growth of the species in question. The second aim was to determine whether any of these substances would be transferred to the BSFL, or even accumulate.

Chapter 3 reports on a second set of experiments which largely mirrored the study on BSFL described in Chapter 2, by assaying the same substances on larvae of a different species: LMW. Mealworm larvae were exposed to known concentrations of those insecticides via the diet, after which survival and growth parameters were recorded, and the larval biomass was analysed to check for accumulation. The aims of this study were to test the effects of these substances on this reared species, and to compare similarities and differences in the observed effects between the two reared species BSFL and LMW that are the focus of this thesis.

Chapter 4 presents the results of an experimental study on BSFL which was conducted as a follow-up to the study described in Chapter 2 of this thesis. This study's main hypothesis was that substances with the same MoA would have additive or synergistic effects. The effects of two classes of insecticides (pyrethroids and organophosphates) were tested by exposing BSFL to several feed substrate concentrations of one representative of each respective class, in order to construct concentration-response curves. Subsequently, the representative and two analogue substances of each class were tested at the same concentration to compare their effects.

Chapter 5 details the results of a long-term study during which two generations of LMW were chronically exposed to insecticides in the feed substrate. In addition to reductions in growth (yield), certain insecticides are known to cause sub-lethal effects that affect reproduction and we hypothesised that this could also be the case for commercially reared insects. To this end, LMW were exposed to two substances that had been found to cause significant reductions in yield in the study described in Chapter 3 of this thesis.

Finally, Chapter 6 (General discussion) provides a synthesis of all experimental results described in the previous chapters. The most important experimental results on the effects of insecticide residues on reared insects are highlighted and placed in context of the relevant recent literature on reared insects and insecticides. Recommendations for follow-up research are provided, as well as implications of current results for insect producers and policy makers.

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Chapter 2

Effects of insecticides on mortality, growth and bioaccumulation in black soldier fly (*Hermetia illucens*) larvae

(Hermetia illucens larvae. Photo by author.)

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Abstract

Residues of persistent insecticides may be present in the substrates on which insects are reared for food and feed, which may affect insect growth or survival. In addition, insecticidal substances may bio-accumulate in reared insects. The objective of this study was to assess potential effects of selected insecticides on the growth and survival of black soldier fly larvae (BSFL, *Hermetia illucens*) and on their safety when used as animal feed. Six insecticides (chlorpyrifos, propoxur, cypermethrin, imidacloprid, spinosad, tebufenozide) with different modes of action were tested in two sequential experiments. Cypermethrin was also tested with the synergist piperonyl butoxide (PBO). Standard BSFL substrate was spiked to the respective maximum residue level (MRL) of each insecticide allowed by the European Union to occur in feed; and BSFL were reared on these substrates. Depending on the observed effects in the first experiment, spiked concentrations tested in the second experiment were increased or reduced. At the concentrations applied (1 and 10 times MRL), three of the six tested substances (chlorpyrifos, propoxur, tebufenozide) did not affect the survival or biomass growth of BSFL, compared to the control (non-spiked) treatments. At MRL, imidacloprid stimulated the growth of BSFL compared to the controls. Spinosad and cypermethrin at the MRL level negatively affected growth and survival. The effects of cypermethrin appeared to be augmented by addition of PBO. A mean bio-accumulation factor of ≤0.01 was found in both experiments for all substances–except for cypermethrin, which was comparatively high, but still below 1 (0.79 at 0.1 mg/kg). The lack of accumulation of insecticides in the larvae suggests that there is no risk of larval products being uncompliant with feed MRLs. However, we conclude that insecticides present in substrates may affect growth and survival of BSFL. More research on a larger variety of substances and insect species is recommended.

Introduction

Insects as food and feed are increasingly seen as a commercially viable mini-livestock alternative to conventional livestock (Van Huis et al., 2013). Larvae of the black soldier fly (BSFL, *Hermetia illucens* (L.); Diptera: Stratiomyidae) had primarily been seen as a pest species (Tomberlin et al., 2002), but have recently received increased attention given their ability to consume and convert a wide variety of organic waste, including vegetable and animal tissue, and manure (Bosch et al., 2019; Gold et al., 2018). However, several safety aspects, including the effects of contaminants that could be present in the feed substrates, have received less attention. One concern is that bio-accumulation of contaminants or chemical residues could result in exceedance of legal limits, or even present a safety risk, when insects–exposed via the substrate on which they are reared–are consumed as food or feed (EFSA, 2015). Synthetic insecticides are an important category amongst potential contaminants that was highlighted for additional research (EFSA, 2015; Van der Fels-Klerx et al., 2018). Moreover, since persistent synthetic insecticides act against all insects, residues of these insecticides present in the substrate may cause (sub-)lethal effects in insects produced for food and feed, even at concentrations in the feed substrate that comply with existing legal limits.

The legal framework for pesticides in the European Union (EU) consists of Regulation (EC) No 1107/2009 concerning the placing of plant protection products [i.e. pesticides] on the market, and Regulation (EC) No 396/2005 on maximum residue levels (MRLs) of pesticides in or on food and feed of plant and animal origin. Maximum levels for certain substances, primarily those prohibited from use in the EU, are set at the lower limit of analytical quantification (LOQ). A pesticide is only approved after risk assessment on its effects on honeybees (*Apis mellifera* L.; Hymenoptera: Apidae), *Bombus* spp. (Hymenoptera: Apidae) and solitary bees has been performed; and it has been concluded that its use would result in negligible exposure of honeybees, and that there will be no unacceptable acute or chronic effects on colony survival and development (Regulation (EC) No 1107/2009, Annex II, section 3.8.3; (EFSA, 2013)). Since insects intended for food or feed are farmed animals (Regulation (EU) No 2017/893), the substrates on which they are reared also must comply with the legal frameworks for pesticides and animal feed (Article 3 of Regulation (EC) No 1069/2009). However, we are not aware of any regulations that consider the potential effects of insecticide residues on insects reared for food and feed in the approval and MRL-setting procedures.

Research on the effects of pesticides on insects has largely focused on two categories of species. Firstly, much research has been done on the eco-toxic effects of insecticides on beneficial species, in particular honeybees due to its well-studied biology, and the mentioned legal requirements for (re-)approval of pesticides (Desneux et al., 2007). A second strand of research has focused on pest species as target of the tested substances (Guedes et al., 2016). However, dose-response relationships are in principle species-

specific (Sanchez-Bayo, 2012), preventing the extrapolation of (sub-)lethal effects of insecticides on other species to BSFL.

The literature on the impact of pesticides on growth and survival of BSFL specifically appears to be relatively scarce. Recent controlled feeding studies on the effects of pesticide residues in the substrate of BSFL have been performed by Tomberlin et al. (2002) (cyromazine, pyriproxifen, λ-cyhalothrin, permethrin), Lalander et al. (2016) (azoxystrobin, propiconazole), and Purschke et al. (2017) (chlorpyrifos, chlorpyrifos-methyl, pirimiphos-methyl). In none of the studies on BSFL mentioned, accumulation of pesticides was observed, and the tested substances did not appear to negatively affect BSFL at concentrations in the feed at or below the MRL.

The primary aim of this study was to perform exploratory research on the effects of a variety of insecticides on the larval stage of *Hermetia illucens*. We hypothesized that residues of tested substances, when present in the feed substrate at concentrations equal to the legal limit in the EU, would affect the growth or survival of BSFL negatively; and that the substances would possibly bio-accumulate in the larvae, resulting in food and feed safety issues. The focus of the experiments was specifically on the larval stage (day 7–14) that is most interesting for commercial rearing of BSFL for feeding purposes.

Methodology

Choice of pesticides

Pesticides targeted at insects (insecticides) can be classified into different groups according to their chemical class and mode of action (MoA). The Insecticide Resistance Action Committee (IRAC) developed a classification scheme in which the insecticide target site and the physiological functions affected, i.e. nerve and muscle, growth, respiration, or mid-gut, were used as classification criteria (Sparks & Nauen, 2015; IRAC, n.d.).

The insecticides to be investigated in this study were chosen using a step-wise approach. A longlist of 54 insecticides, acaricides, and nematicides was created from a total of 527 compounds (including isomers) listed in the database of the Dutch National Monitoring Programme on contaminants in feed and food. The 54 insecticides included in the longlist were detected (i.e. analytical result > LOD) in feedstuffs and edible insects in The Netherlands, as based on data from the Dutch National Control Programme Animal Feed in 2015–2016, and data supplied by three major insect producing companies in The Netherlands. Of these, 16 substances were isomers of other substances and were therefore disregarded. Of the remaining 38 substances, one insecticide was selected from each chemical class/MoA as classified by IRAC (Sparks & Nauen, 2015). In case of multiple insecticides per class, the substance with the highest number of entries in the Rapid Alert System for Food and Feed (RASFF) database for the years 2015–2017 was selected. In total, six insecticides and one synergist were selected. These selected compounds, their class and mode of action, are summarized in Table 1.

Table 1: Selected insecticides including class, mode of action, and maximum residue level (MRL) in the EU for feed, or maize specifically.

Substance name	**Class**	**Mode of Action**	**MRL[1] (mg/kg)**
Chlorpyrifos	Organophosphates	Acetylcholinesterase (AChE) inhibitors	0.05
Propoxur	Carbamates	Acetylcholinesterase (AChE) inhibitors	0.05*
Cypermethrin	Pyrethroids	Sodium channel modulators	0.3
Imidacloprid	Neonicotinoids	Nicotinic acetylcholine receptor (NAchR) competitive modulators	0.1
Spinosad	Spinosyns	Nicotinic acetylcholine receptor (NAchR) allosteric modulators – site I	2.0
Tebufenozide	Insect growth regulators (IGRs)	Ecdysone receptor agonists	0.05*
Piperonyl butoxide	Synergist	Synergist	Not a plant protection product; no MRL

1: For feed (Directive 2002/32/EC), if available, or maize (Reg. (EC) No 396/2005).
**: Indicates lower limit of analytical determination.*

The insecticides methoxychlor (MoA: sodium channel modulator) and various isomers of DDT were detected (>LOQ) in feed materials in the Netherlands National Control Programme Animal Feed 2015–2016. However, these have not been included in the final list because they have been banned in the EU since 2002 (Regulation (EC) No 2076/2002) and exposure is therefore expected to be incidental only. More RASFF notifications have been filed for the neonicotinoid acetamiprid (189) than for imidacloprid (76). However, due to imidacloprid's higher toxicity and increased attention it has received recently due to its sub-lethal effects on honeybees (see e.g. EFSA (2018)), it was decided to select imidacloprid instead of acetamiprid. The impact of the organophosphate chlorpyrifos on BSFL was found to have no effect on survival and growth, and not to bio-accumulate (0.4 mg/kg) by Purschke et al. (2017); its inclusion in this study was aimed to verify their results on the BSF strain we tested. At the time that the experiments reported here were conducted (September 2018), chlorpyrifos was still permitted to be used in the EU. In 2019, the European Food Safety Authority (EFSA) concluded that the genotoxic potential of chlorpyrifos could not be excluded, and it was therefore recommended that its approval would not be renewed (EFSA, 2019). With the substance's authorisation expiring after 31 January 2020, this non-renewal effectively prohibited the use of this pesticide after this date (Regulation (EU) 2020/18).

Piperonyl butoxide (PBO) is not an insecticide in the strict sense of the word: it is primarily used as a synergist. Some studies suggest that PBO may have intrinsic insecticidal properties by acting as a juvenile hormone mimic (preventing adult development) (Srivastava & Gilbert, 1969). However, it is generally used at sub-lethal levels to enhance the effects of mainly pyrethrin and synthetic pyrethroids (WHO,

2011). In this study, PBO was therefore combined with cypermethrin in a single treatment, in addition to a treatment containing only PBO. No MRL has been set for PBO in the EU because it is not registered as a plant protection product (Regulation (EC) No 2016/2288).

Feed preparation

The feed preparation and experimental set-up were largely based on Camenzuli et al. (2018). A standard substrate containing primarily wheat, potato, and yeast used for commercial rearing of BSFL was obtained from Bestico B.V. (Berkel en Rodenrijs, The Netherlands), where the experiments were conducted. Two experiments were performed with a number of treatments: in experiment 1 (Exp. 1), individual batches of feed were spiked to the European MRL for that insecticide in feed specifically ('1*MRL') as defined in Directive 2002/32/EC), or for maize (as defined in Regulation (EC) No 396/2005), see Table 1. Depending on the effects of the insecticides at these levels in terms of BSFL growth and mortality as compared to control, higher or lower concentrations were used in experiment 2 (Exp. 2, '+/-*MRL'; see below). Due to the short shelf-life of the substrate, spiked concentrations could not be verified prior to the experiment, nor checked for the presence of insecticides prior to spiking.

The analysed substances, their purity, solvent, and suppliers, are presented in Table 2. PBO was spiked in two treatments: one treatment in which it was spiked together with the pyrethroid cypermethrin at a ratio common in commercial formulations, of 20:1 (Osimitz, 2010), and one treatment containing only PBO–at the same concentration as in the other treatment. The spinosad treatment was a mixture of spinosyns A and D at a ratio of 74.2:22.3. In Exp. 2, the spiked concentration for cypermethrin was 1/3 * MRL (this also affected the PBO level since that was 20 * the level of cypermethrin).

Table 2: Insecticides tested, their intended spiked concentration in BSFL-feed, solvent, purity and suppliers.

Substance	**Intended spiked concentration (mg/kg)**		**Solvent**	**Purity (%)**	**Supplier**
	Exp. 1	**Exp. 2**			
Chlorpyrifos	0.05	0.5	MeOH	99.3	Sigma-Aldrich[1]
Propoxur	0.05	0.5	MeOH	99.9	HPC[2]
Imidacloprid	0.1	1.0	MeOH	98.7	HPC[2]
Spinosad	2.0	0.2	MeOH	96.6	HPC[2]
Tebufenozide	0.05	0.5	ACN	99.9	Sigma-Aldrich[1]
Cypermethrin	0.3	0.1	ACN	99.7	HPC[2]
Piperonyl butoxide (PBO)	6.0	2.0	ACN	92.5	Dr. Ehrenstorfer[3]
Cypermethrin + PBO	0.3 + 6.0	0.1 + 2.0	ACN	99.7 + 92.5	HPC[2] + Dr. Ehrenstorfer[3]

1: Sigma-Aldrich Chemie N.V., Postbus 27, 3330 AA Zwijndrecht, The Netherlands
2: HPC Standards GmbH, Am Wieseneck 7, 04451 Cunnersdorf, Germany
3: LGC Standards GmbH, Mercatorstrasse 51, 46485 Wesel, Germany

The spiked concentration of spinosad in Exp. 2 was 0.1 * MRL. Lower levels for these two insecticides in Exp. 2 relative to Exp. 1 were chosen due to the higher mortality observed in the spinosad treatment. For the other compounds (chlorpyrifos, propoxur, imidacloprid, and tebufenozide), the spiked concentration in Exp. 2 was 10 times the spiked concentration of Exp. 1, i.e. 10 * MRL.

In addition to the spiked feed treatments, three control treatments were used in Exp. 1: one blank, and two solvent controls (either acetonitril (ACN) or methanol (MeOH)). In Exp. 2, only the two solvent controls containing ACN and MeOH controls were used and the blank control was omitted because differences between the three controls in Exp. 1 were not significant.

Per treatment, 400 g (± 1 g) of feed was weighed in a 1 L beaker. This feed was spiked with the selected insecticide (dissolved in the respective solvent) to the desired concentration. The feed was then homogenized with a Bosch ErgoMixx hand-mixer (Robert Bosch Hausgeräte GmbH, Munich, Germany). From each beaker, 50 g (± 0.25 g) of spiked feed was transferred to each of the three containers in which the larvae would be placed. In addition, 1 g (± 0.1 g) of spiked feed was transferred into test tubes to verify the homogeneity of the insecticides in the spiked feed; and 50 g was placed in a separate container to verify the concentration. See section 2.4 for a description of the quality control (QC) parameters of these analyses.

Animal procedures

Treatments were performed in triplicate. Per replicate, 100 seven-day-old larvae (post-hatching) were reared on 50 g of feed. The containers used in this trial were cylindrical (diam. 100 mm, height 40 mm) and a circular area (diam. 40 mm) in the centre of the lid consisted of fine mesh to allow for ventilation (SPL Life Sciences Co., Ltd., Gyeonggi-do, South Korea). Containers were distributed over trays; these trays were stacked and placed in a climate chamber (set at 28°C and 60% RH). The larvae were reared for another seven days until day 14 post-hatching, which was in line with the commercial practices of BSFL rearing at Bestico (Berkel en Rodenrijs, The Netherlands), where the experiments were conducted.

After seven days, the larvae were separated from the residual material (RM), which consisted of larval excreta and residual feed. The larvae were counted by manually removing them from the container, using metal tweezers. Dead larvae that appeared desiccated or immobile, were separated from live larvae. Larvae for which it was doubted if they were dead or displayed thanatosis were provisionally placed with the dead larvae until subsequent steps had finished for that replicate (approximately 2 min.). Larvae that had resumed moving were placed with the respective live larvae; otherwise they would be presumed dead. Larvae and residual materials were weighed. Larvae collected from the substrate were cleaned by depositing them in a standard plastic kitchen sieve, cleaned by rinsing them with running water to remove adhering residual material, and then dried gently using a paper towel. Between treatments,

equipment (sieves and forceps) was rinsed and dried. Finally, the larvae were killed by freezing and kept frozen (at -18° C) until subsequent chemical analyses. Residual material was collected, weighed, and then stored in a clean plastic container at -18° C until analysis.

Chemical analyses

Concentrations of tested insecticides in the substrate, larvae, and residual material were analysed using liquid chromatography-mass spectrometry (LC/MS-MS).

Extraction

For extraction of the active compounds from the larvae, the following procedure was followed. Frozen sample material, 1.0 g (± 0.05 g), was weighed into a tube. This was diluted by adding 5 ml of milliQ and 5 ml of acetonitrile + 1% acetic acid. The sample was homogenised by using an ultra-turrax machine until finely ground; 0.5 g of sodium acetate (ACS reag. Ph Eur. Emsure Merck) and 2 mg $MgSO_4$ (GPR Rectapur VWR chemicals) was added, followed by vortexing for 30 s and centrifugation for 5 min at 3600 rpm (VWR Microstar 17). A volume of 2.5 ml was evaporated to < 0.5 ml and acetonitrile (Biosolve HPLC Supra Gradient 01203502) was added to bring the sample volume to 0.5 ml. This sample was mixed with 75 mg $MgSO_4$, 12.5 mg C18 (Bakerbond Octadecyl (C18) 40 µm, JT Baker), 125 mg PSA (Bondesil-PSA 40 µm Agilent Technologies) + 25 µl 2 µg/ml PCB 198 (Ultra Scientific RPC-075S (diluted from 100 µg/ml > 2 µg/ml in hexane)), and vortexed for 30 s, and finally centrifuged for 5 min at 13.000 rpm (Thermo Scientific SL 40R Centrifuge). This was transferred to an LC vial and diluted where necessary. For extraction of the residual material, the same procedure was followed except that the homogenisation step using an ultra-turrax machine was replaced with 30 min of end-over-end mixing.

Analyses

A Waters ultra-high-performance liquid chromatography (UPLC) system (Waters, Etten-Leur, The Netherlands) and an Applied Biosystems Qtrap 6500 MS (Applied Biosystems Bleiswijk, The Netherlands) equipped with an electrospray (ESI) source were used. Separation was performed on a Acquity UPLC HSS T3, 1.8 µm, 2.1 x 100 mm column (Waters, Etten-Leur, The Netherlands) using a flow rate of 0.4 mL/min. The column temperature was maintained at 40°C. Eluent A was water (purified using a Milli QR system with a minimal specific resistance of 10 MΩ.cm-1, or water of a similar quality) containing 5 mM ammonium formate (> 99%, Sigma-Aldrich 17843) and 0.1% (v/v) formic acid 98–100% (EMSURE® ACS, Reag. Ph Eur (VWR 1.00264.1000)). Eluent B was water/methanol 5/95 (methanol: Biosolve 13683502 Absolute HPLC Supra Gradient) (v/v) containing 5 mM ammonium formate and 0.1% (v/v) formic acid. Total runtime was 12 min. The UPLC gradient started with 100% A for 1 min, was linearly increased to 100% B over 5 min, and kept at this percentage for 3 min. Finally, the gradient was switched to 100% A again over 0.5 min and equilibrated for 2.5 min before the next injection took place. The injection volume was 10 µL.

MS/MS conditions

ESI-MS/MS was performed using multiple reaction monitoring (MRM) in positive mode. Acquisition was done with 10 ms dwell time. The settling time and MR pause time was set to 5 ms. The number of data points across the peaks was at least eight. The settings of the ESI-source were as follows: source temperature 500°C, curtain gas 35 psi, source gas 1 50 psi, source gas 2 50 psi, ion spray voltage + 4000 V and collision gas (nitrogen) medium. The analyte-dependent parameters declustering potential (DP), collision energy (CE) and cell exit potential (CXP) are listed in S1 Table.

Quality control

Quality control (QC) was performed by spiking blank samples with active substances in the range of 1–100 µg/kg. Results for these analyses can be found in S2 Table (substrate), S3 Table (residual material) and S4 Table (substrate). For the residual material and larvae, the QC was performed for both experiments; for the substrate this was performed for Exp. 1.

Calculations

Based on concentrations in the three matrices (substrate, larvae, and residual material), bio-accumulation and mass balance calculations were performed. The bioaccumulation factor (BAF) was defined as the concentration of the analysed insecticide in the larvae, divided by the concentration of that compound in the feed (Van der Fels-Klerx et al., 2016). Bio-accumulation calculations for Exp. 1 were based on the analysed concentration in the substrate, whereas for Exp. 2, bio-accumulation calculations were estimates based on the spiked concentration in the substrate. In case the concentration in the larvae was below the limit of quantification (< LOQ), the BAF was not calculated.

For Exp. 1, mass balance calculations were performed in order to compare the insecticide levels pre- and post-experiments. The analysed weight (mg) of quantified substances in (i) the larvae and (ii) residual matter, post-experiments, was determined and expressed as a percentage of the measured weight (mg) of analysed substances in the feed, pre-experiments. As with bio-accumulation, the mass of substances in the larvae and residual material expressed as a percentage of the mass in the substrate was not calculated if the concentration in the respective matrix could not be quantified (concentration below LOQ). The sum of the insecticide amount (in weight units) in the larvae and residual material post-experiment being equal to the amount in the feed substrate pre-experiment implies that the total amount of spiked substance was recovered, and that no metabolic conversion has taken place. It was assumed that the concentration as determined in the sample of larvae or residual material was homogenously distributed and representative for the concentration in the entire replicate from which the sample was taken. For Exp. 2, mass balance calculations were based on the intended spiked concentration in the substrate.

Statistical analyses

For the statistical analyses, the software SPSS Statistics for Microsoft Windows (version 25.0.0.2, IBM Corp., Armonk, NY, United States) was used. Because the treatments were performed in triplicate, tests on conformity to a distribution type were not warranted and non-parametric statistical tests were therefore used to determine statistical significance of findings.

To test whether the solvents (ACN and MeOH) had a significant effect on growth or survival compared to the blank control in Exp. 1; the distributions of the three control treatments were compared using a Kruskal-Wallis test ($\alpha = 0.05$). If this was not the case, the controls in Exp. 1 ($n = 9$) and Exp. 2 ($n = 6$) were pooled for further statistical comparison with treatments containing active substances in each of the respective experiments.

For Exp. 1, the effects of all active substances on survival and growth of the larvae were tested for significant differences among treatments by using a Kruskal-Wallis test ($\alpha = 0.05$). In Exp. 2, compared to Exp. 1, some active substances were used at higher concentrations (10x), and some were used at lower concentrations (1/3 and 1/10)– depending on the results of Exp. 1. The treatments in Exp. 2 containing active substances that were present in concentrations 10x that tested in Exp. 1 (chlorpyrifos, propoxur, imidacloprid, tebufenozide), were tested together with the pooled controls for significant differences by using a Kruskal-Wallis test ($\alpha = 0.05$). This was also done for the treatments containing active substances present at 1/3 the concentration of Exp. 1 (cypermethrin, PBO, cypermethrin + PBO). For the single treatment in Exp. 2 that contained an active substance present at 1/10 the concentration of Exp. 1 (spinosad), significance of differences with the pooled controls was tested by using a Mann-Whitney U test ($\alpha = 0.05$).

For both Exp. 1 and Exp. 2, if differences between treatments and controls in the Kruskal-Wallis test were significant ($P < 0.05$), then each treatment was compared separately to the grouped controls by using a Mann-Whitney U test. Because this post-hoc test involved multiple comparisons, a lower α value ($\alpha = 0.01$) was used.

Results

Quality control

In the substrate of Exp. 1, the recovery of substances was within the acceptable range of 70–120% (DG SANTE, 2017); with the exception of propoxur (138%). These samples complied to the repeatability criteria (RSD ≤ 20%) though, and it was therefore decided to correct the concentrations for recovery. In the residual material, the average recovery of all compounds complied to the acceptability criteria of 70–120%, with the exception of spinosad (47% in Exp. 2) for which concentrations were accordingly corrected. The exact reason for this could not be identified, but this may have been due to the higher spinosad concentration in the samples, when compared to the

concentration in the QC samples. In BSF larvae samples, the average recovery of all compounds complied to the acceptability criteria of 70–120% with the exception of PBO (64% in Exp. 1). This was likewise assumed to be due to differences between the QC and spiked concentrations. All compounds complied to the repeatability criteria (RSD ≤ 20%), with the exception of tebufenozide with an RSD of 33%. This did not affect the results since no quantifiable amount of tebufenozide was detected in the triplicate samples.

Larval survival

Data of larval survival are shown in S5 Table, for both experiments. In two replicates in Exp. 1 (tebufenozide (n = 104), blank control (n = 105)) survival was > 100%, which was assumed to be due to a counting error. These outliers were therefore corrected by assuming survival was 100%. The mean larval survival of all replicates of the three control treatments in Exp. 1 (ACN, MeOH, blank) was 99.4 ± 0.7% and not statistically different between the three control treatments (P = 0.056), we therefore pooled the control groups for subsequent statistical tests. Because the solvents did not have a significant effect on survival in Exp. 1, the controls were also pooled in Exp. 2.

In Exp. 1, BSFL survival was statistically different between treatments (P = 0.004). The results for survival in Exp. 1 are shown in Fig 1. For most of the investigated insecticides (chlorpyrifos (P = 1.000), propoxur (P = 0.282), imidacloprid (P = 0.727), tebufenozide (P = 1.000), PBO without cypermethrin (P = 0.282)), differences in survival between these treatments and the control were not significant. However, spinosad (P = 0.009), cypermethrin (P = 0.009), and cypermethrin mixed with PBO (P = 0.009) reduced the survival of the larvae significantly as compared to the control in Exp. 1. The negative effect of cypermethrin on survival appeared to be enhanced by the addition of PBO, as compared to the treatment with cypermethrin only, but the number of replicates was too small to determine the significance of this difference using the Mann-Whitney U-test (n1 = n2 = 3).

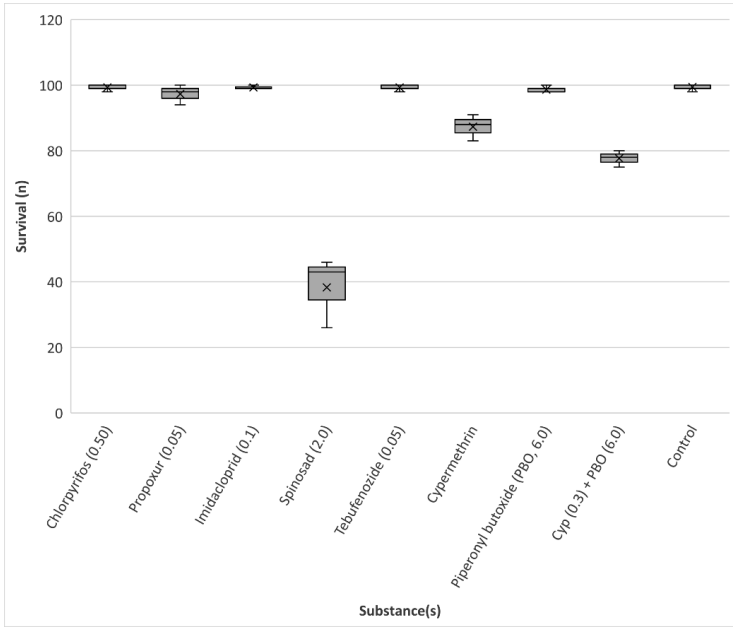

Figure 1: Box-plot of survival of black soldier fly larvae (Hermetia illucens) reared on substrates contaminated with different insecticides in Exp. 1. Results of the three control treatments have been grouped together. The concentration spiked in the substrate is indicated behind the name of the substance (mg/kg).

In Exp. 2, differences between the controls and treatments containing active substances present in the substrate at 10x the concentration of Exp. 1 (chlorpyrifos, propoxur, imidacloprid, tebufenozide) were not significant (P = 0.155). This was also not significant for the substances present at 1/3 the concentration (cypermethrin, PBO, cypermethrin + PBO) (P = 0.356), nor for spinosad present at 1/10 the concentration (P = 1.000).

Larval growth

Data on the increase in biomass of the larvae are shown in S5 Table, for Exp. 1 and 2. The mean larval growth of all replicates of all control treatments in Exp. 1 was 10.86 ± 0.30 g and not statistically different between the three control treatments (P = 0.301), we therefore pooled the control groups for subsequent statistical tests. Because the solvents did not have a significant effect on survival in Exp. 1, the controls were also pooled in Exp. 2.

As with survival, differences in larval growth were significant in Exp. 1 (P = 0.002). Results for the increase in biomass in Exp. 1 are shown in Fig 2; chlorpyrifos (P = 1.000), propoxur (P = 0.373), tebufenozide (P = 0.864), and PBO without cypermethrin (P = 0.864) did not affect larval biomass increase when compared to the control treatments (P > 0.01). However, imidacloprid significantly enhanced larval biomass growth (P = 0.009). The same insecticides that reduced larval survival (spinosad (P = 0.009), cypermethrin (0.009), and cypermethrin with PBO (P = 0.009)), also

significantly reduced biomass increase compared to the control ($P \leq 0.01$). As seen in the results for survival, PBO tended to enhance the negative effects of cypermethrin on growth but a test on significance could not be done due to low sample size (n1 = n2 = 3).

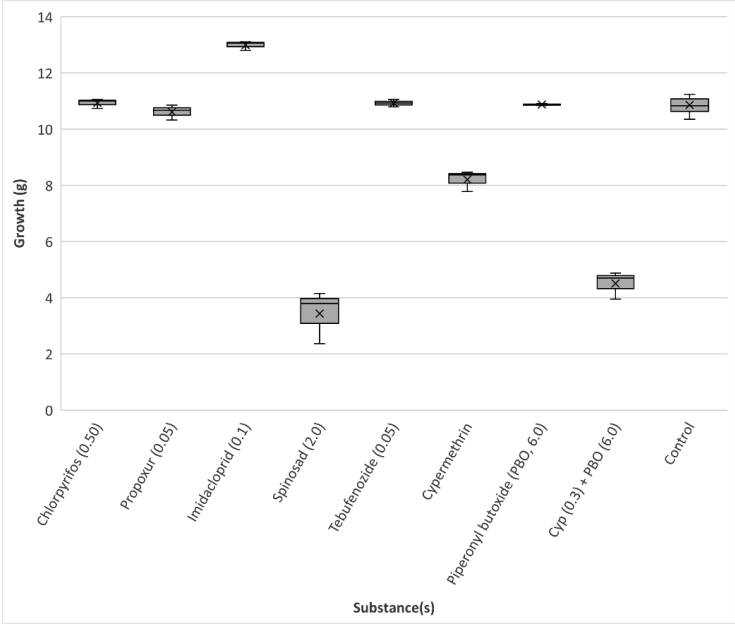

Figure 2: Box-plot of increase in biomass (g) of black soldier fly larvae (Hermetia illucens), reared on substrates contaminated with different insecticides in Exp. 1. Results of the three control treatments have been grouped together. The concentration spiked in the substrate is indicated behind the name of the substance (mg/kg).

As seen for the survival in Exp. 2, differences in growth between treatments was not significant for any of the tested active substances. This was the case for those substances present in the substrate at 10x the concentration of Exp. 1 (chlorpyrifos, propoxur, imidacloprid, tebufenozide; P = 203); 1/3 the concentration (cypermethrin, PBO, cypermethrin + PBO; P = 0.069); and 1/10 the concentration (spinosad; P = 0.381).

Concentrations and bioaccumulation

In the residual material of Exp. 1, cypermethrin (mean 0.035 mg/kg) and PBO (mean 0.013 mg/kg) in control treatments could be quantified in treatments in which it was not spiked. In Exp. 2, the mean concentrations of these two substances were 0.005 and 0.021 mg/kg, respectively. Other pesticide residues were not detected above their respective LOQ in treatments in which they had not been spiked.

The analysed concentrations (mg/kg) of tested compounds in the substrate (Exp. 1), and larvae and residual material (Exp. 1 and Exp. 2) are shown in S6 Table (Exp. 1) and S7 Table (Exp. 2).

Based on the analysed concentrations in Exp. 1, the BAF could be calculated for spinosad, cypermethrin, and PBO (shown in Fig 3). Concentrations of the remaining four substances (chlorpyrifos, propoxur, imidacloprid, tebufenozide) could not be quantified in the larvae, and the BAF was therefore not calculated. In case of cypermethrin, BAF was 0.51 ± 0.08; for other substances the mean BAF was < 0.20. Noteworthy is that the BAF of cypermethrin when mixed with PBO (0.12 ± 0.02) was four times lower than without this synergist (0.51 ± 0.08). Nonetheless, for all tested substances, mean BAF was < 1—signifying that none of these substances accumulated in the larvae.

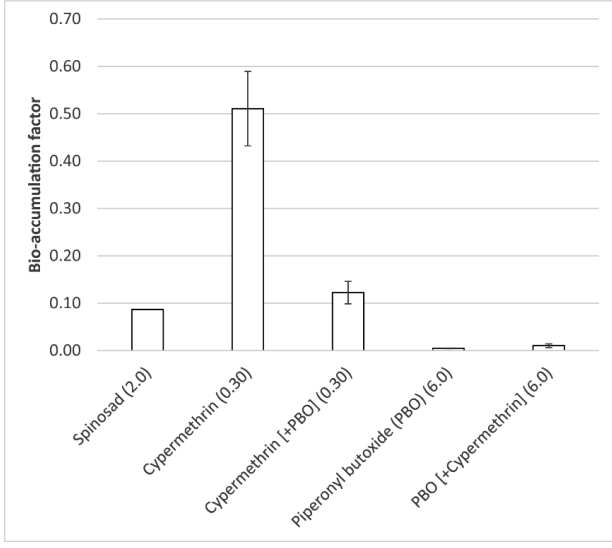

Figure 3: Bio-accumulation factor (mean + SD) of black soldier fly larvae (Hermetia illucens) reared on substrates contaminated with different insecticides in Exp. 1. The concentration spiked in the substrate is indicated behind the name of the substance (mg/kg).

In Exp. 2, the BAF was expressed as the analysed concentration in the larvae relative to the spiked concentration in the substrate. For this experiment, the BAF of cypermethrin was 0.79 ± 0.25 (without PBO) and 0.52 ± 0.19 (with PBO). While the concentration in the larvae could be quantified for the remaining substances (with the exception of propoxur), the mean BAF in Exp. 2 was ≤ 0.03 for each of these substances.

Mass balance calculations were performed for Exp. 1 (shown in Fig 4). As mentioned for the bio-accumulation, concentrations in the larvae could not be quantified for chlorpyrifos, propoxur, imidacloprid, and tebufenozide. In the residual material, the proportion of these substances post-trial was low (≤ 10%, and 20% for chlorpyrifos). Concentrations of spinosad and PBO could be quantified in the larvae, but the contributions to the total post-trial mass of each substance were very low (< 1.0%). The post-trial proportion of PBO in the residual material, relative to the respective total pre-trial mass in the substrate, was ca. seven times higher in the treatment also containing cypermethrin (54.3 ± 6.8%) than in the treatment without cypermethrin

(7.8 ± 1.3%). The opposite was observed for cypermethrin, both in the larvae and the residual material. For both matrices, the proportion in the treatment containing only cypermethrin (larvae: 8.9 ± 1.4%; residual: 121.2 ± 7.5%) were on average higher than for the treatment containing both cypermethrin and PBO (larvae: 1.2 ± 0.3%, ca. 7 times lower; residual: 87.4 ± 6.7% (n = 2), 1.4 times lower). We ascribed the high concentration of cypermethrin in the residual material in Exp. 1 to inhomogeneous distribution of this substance in the matrix.

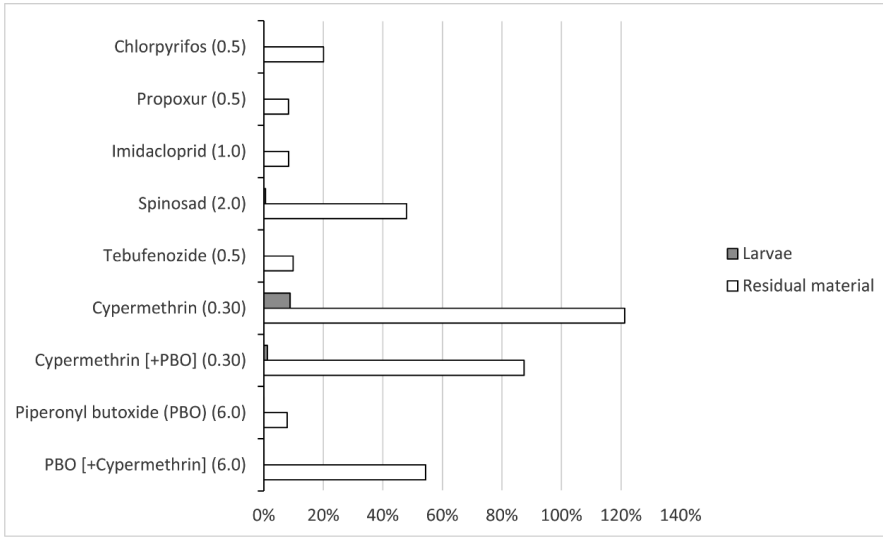

Figure 4: Mass balance (mean + SD) of different insecticides in Exp. 1 (n=3 per treatment). Mass in black soldier fly larvae (Hermetia illucens) and residual material post-experiment expressed as a percentage of the analysed mass in the pre-trial substrate.

In Exp. 2, the mean proportion of substances in the larvae and residual material, as expressed as a percentage of the spiked mass, was low (< 10%) for chlorpyrifos, imidacloprid, tebufenozide, propoxur and PBO with and without cypermethrin. For all these substances, the percentage found back in the larvae was very low (< 1%). The proportion of spinosad post-trial was 13.4 ± 2.9%, with a higher percentage in the residual material (12.9 ± 2.8%) compared to the larvae (0.5 ± 0.2%). The proportion of cypermethrin recovered in the larvae, expressed as a percentage of the spiked amount, was 16.3 ± 5.2% (without PBO) and 10.9 ± 4.2% (with PBO). The concentration of cypermethrin in the residual material in the treatment without PBO was not determined due to a dilution error in sample preparation–but in the treatment with PBO it was also elevated at 22.2 ± 3.3%, compared to the other substances.

Discussion and recommendations

Three of the tested active substances (chlorpyrifos, propoxur, tebufenozide) did not significantly affect growth nor survival, when present in the substrate at concentrations up to 10 times the MRL (0.5 mg/kg, for all three substances). Concentrations of these active substances in the larvae were either below the LOQ or very low, in comparison

to the concentration in the substrate; indicating that bio-accumulation did not occur. For chlorpyrifos, these results confirm the findings of Purschke et al. (2017): in that study it was also concluded that there was no significant effect of chlorpyrifos spiked in the substrate at approximately the same concentration (0.4 mg/kg) on the survival and growth of BSFL, and that this substance did not bio-accumulate (Purschke et al., 2017).

Mass balance calculations showed that total post-trial recovery of these insecticides in the larval biomass and residual material was below 100% in both experiments, compared to what was present in the substrate pre-trials. The mass balance being below 100% suggests that metabolic conversion of the spiked active substances may have occurred to some degree. Since growth and survival of BSFL were unaffected in these treatments, this conversion may have resulted in detoxification of the spiked parent compounds, but additional research on metabolic pathways in BSFL is needed to verify this.

Although we observed no effects on growth and survival of chlorpyrifos, propoxur, tebufenozide on BSFL in the specific developmental phase investigated in this study (7 to 14 days old), additional and/or different effects on adults or younger larvae cannot be excluded. In particular, sub-lethal effects of larval exposure on, inter alia, adult mating behaviour, fecundity, and longevity of adults (Desneux et al, 2007) should be investigated to ensure that these substances do not affect the continuity of a colony, as this could also have major financial consequences for BSFL farmers.

Two of the tested insecticides (spinosad and cypermethrin) negatively affected both the growth and survival of the larvae when present at concentrations equal to the MRL (2.0 and 0.3 mg/kg, respectively). However, when lower concentrations of these substances were used in Exp. 2 (spinosad: 0.2 mg/kg; cypermethrin: 0.1 mg/kg), growth and survival of the BSFL were not affected to a significant degree.

The effects of cypermethrin appear to have been augmented by addition of the synergist piperonyl butoxide (PBO). In general, PBO's mode of action–when combined with a pyrethroid such as cypermethrin–is that it inhibits microsomal oxidase enzymes from detoxifying the active substance (Moores et al., 2009). Therefore, a higher concentration of cypermethrin would be expected to remain unmetabolized in the insects when they are also exposed to PBO, than if that synergist is not added. However, the opposite was observed in this study. The mean proportion of cypermethrin in the larvae recovered post-trial (16.2 ± 5.2%) was highest in the treatment without PBO in Exp. 2 in which survival and growth were not significantly affected–but lowest in the treatment containing both cypermethrin and PBO in Exp. 1 (1.2 ± 0.3%), in which survival and growth were lowest of the four tested treatments containing cypermethrin in the two experiments. These results taken together suggest that the interaction between cypermethrin and PBO that caused the reduced growth and survival, is linked to an as yet unknown metabolization of these substances to potentially more toxic metabolites. Additional research on the effects of cypermethrin in combination with PBO on BSFL is therefore recommended: to statistically validate the synergistic effects of

PBO by using a higher number of replicates, and to determine the metabolic products and pathways that underlie these effects.

It is plausible that insecticides with the same MoAs as spinosad (nicotinic acetylcholine receptor (nachr) allosteric modulators—site I) and cypermethrin (sodium channel modulators) (IRAC, n.d.) may also negatively affect growth and survival of BSFL. An insecticide with the same MoA as spinosad is spinoteram; examples of other pyrethroids with the same MoA as cypermethrin include deltamethrin, cyfluthrin, and various isomers of these compounds (IRAC, n.d.). Based on the findings presented here, we would advise prioritization of these substances in future studies on the effects of insecticide residues on BSFL.

Imidacloprid had a significantly positive effect on the increase in biomass in Exp. 1, compared to the control treatments. The positive effects of imidacloprid may be ascribed to insecticide-induced hormesis, a dose-dependent phenomenon in which low doses of a substance may incite stimulatory effects while higher doses are toxic (Guedes & Cutler, 2014). Effects may include, amongst others, increase in birth rate, growth rate, and percentage of reproductive adults (Cutler & Guedes, 2017; Forbes, 2000). No prior studies published in the scientific literature on the effects of imidacloprid specifically on BSFL could be found, but there is some literature available on beneficial effects of sublethal doses of imidacloprid on other species. For instance, direct and intra-generational stimulatory effects on reproduction have been observed for sub-lethal doses of imidacloprid on green peach aphid (*Myzus persicae* Sulzer; Hemiptera: Aphididae) (Ayyanath et al., 2013; Cutler et al., 2009; Yu et al., 2010) and melon aphid (*Aphis gossypii Glover*; Hemiptera: Aphididae) (Ullah et al., 2019). Furthermore, hormetic effects were observed for reproduction and immature development duration of *Aphis glycines Matsumura* (Hemiptera: Aphididae) (Qu et al., 2015); as well as an increase in reproductive fitness of male neotropical stink bugs (*Euschistus heros F.*; Heteroptera: Pentatomidae) (Haddi et al., 2016). It should be noted that the sublethal dose of imidacloprid that was observed to stimulate growth of the BSFL in this experiment, may also result in inhibitory effects on, for instance, pupation or reproduction, or possibly intra-generational effects (Cutler & Guedes, 2017). Therefore, additional research on the dose-response relationship of imidacloprid and BSFL is highly recommended.

The findings from this study have implications for BSFL farmers and policy-makers. Insecticide MRLs depend on the feed material: for instance, the MRL of cypermethrin in maize that was used in this study is 0.3 mg/kg–while it is 2.0 mg/kg in wheat. Consequently, higher concentrations are permitted in wheat, which could have even more devastating effects on survival and growth than what was found in our experiments. Cypermethrin and spinosad are insecticides approved for use in the EU. Feed intended for insects with concentrations of these compounds at or slightly below the MRL may be legally put on the market–but at these concentrations they may pose a health risk to BSFL, and thus present a commercial risk for the insect farmer. It is

recommended that BSFL farmers target cypermethrin and spinosad in analyses of their incoming feed streams to ensure the safety of these materials before use as feed substrate. We also advise vigilance in checking incoming feed materials for insecticides with the same MoAs–until further research can rule out negative effects of these other insecticides at legally allowed levels. The results of this study indicate that the potential effect of insecticide residues on farmed insects is a factor that should be taken into consideration when insecticides are approved or MRLs are set.

Conclusion

Three of the six (chlorpyrifos, propoxur, and tebufenozide) tested insecticides tested at concentrations equal to the respective MRL and 10x that concentration did not affect survival or biomass growth of BSFL. Cypermethrin and spinosad reduced survival and increase of biomass of the BSFL in this experiment. The synergist piperonyl butoxide (PBO) alone did not affect BSFL at concentrations up to 6.0 mg/kg–but when combined with cypermethrin, the negative effects of cypermethrin on growth and survival tended to be enhanced. To validate this, the experiment should be repeated with a higher number of replicates. Imidacloprid stimulated the growth of BSFL, but the exact underlying mechanism for this is unclear and requires more research.

None of the tested substances accumulated in BSFL. This suggests that rearing BSFL on feed containing these insecticides at concentrations equal to the respective MRLs, will not result in exceedance of those limits in BSFL at the point of harvest–notwithstanding the aforementioned risks for survival and growth.

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Supplementary materials

Table S1. MS/MS conditions.

Q1	Q3	Substance ID	DP	CE	CXP
349.9	96.9	Chlorpyrifos	41	41	20
349.9	198	Chlorpyrifos 2	41	25	20
359.9	98.9	Chlorpyrifos D10 1	41	41	20
433.1	191	Cypermethrin	21	21	25
433.1	127	Cypermethrin 2	21	39	25
256.1	175.1	Imidacloprid	41	25	12
256.1	209.1	Imidacloprid 2	41	23	14
260.1	179.1	Imidacloprid D4	41	25	12
260.1	213.1	Imidacloprid D4 2	41	23	14
356.2	177.1	Piperonyl butoxide	31	19	25
356.2	119.1	Piperonyl butoxide 2	31	47	25
210.2	111	Propoxur	61	19	14
210.2	168.1	Propoxur 2	61	13	4
217.2	112	Propoxur D7	61	19	14
732	142	Spinosyn A	186	41	20
732	98	Spinosyn A 2	186	93	22
746	142	Spinosyn D	186	43	20
746	99	Spinosyn D 2	186	75	12
353	133	Tebufenozide	86	33	20
353	297	Tebufenozide 2	86	13	32
362	133	Tebufenozide D9	86	33	20

Legend: Q1: first quadrupole; Q3: third quadrupole; DP: declustering potential; CE: collision energy; CXP: cell exit potential

Table S2: Quality control results substrate for Exp. 1.

Exp. 1 (1*MRL)				
Substance name	**Average recovery**	**RSD [a]**	**n**	**Spike range (mg/kg)**
Chlorpyrifos	101%	19%	3	0.005-0.10
Cypermethrin	91%	0.4%	2	0.05-0.10
Imidacloprid	86%	26%	3	0.005-0.10
PBO	115%	6.8%	2	0.005-0.10
Propoxur	138%	2.3%	3	0.005-0.10
Spinosad	85%	7.6%	3	0.005-0.10
Tebufenozide	89%	7.8%	3	0.005-0.10

Legend: a: relative standard deviation

Table S3: Quality control results residual material for Exp. 1 and 2.

Exp. 1 (1*MRL)				
Substance name	**Average recovery**	**RSD [a]**	**n=**	**Spike range (mg/kg)**
Chlorpyrifos	103%	4.6%	8	0.005
Cypermethrin	#	#	#	0.005
Imidacloprid	97%	11%	8	0.005
PBO	#	#	#	0.005
Propoxur	98%	4.4%	8	0.005
Spinosad	92%	23%	5	0.005
Tebufenozide	111%	15%	8	0.005
Exp. 2 (+/-*MRL)				
Chlorpyrifos	84%	7.6%	4	0.005-0.025
Cypermethrin	79%*	31%	2	0.005-0.025
Imidacloprid	101%	9.9%	4	0.005-0.025
PBO	76%	40%	2	0.005-0.025
Propoxur	103%	6.1%	4	0.005-0.025
Spinosad	47%	9.8%	3	0.005-0.025
Tebufenozide	79%	23%	4	0.005-0.025

*Legend: *: Solvent only; #: Positive blank; a: Relative standard deviation*

Table S4: Quality control results larvae for Exp. 1 and 2.

Exp. 1 (1*MRL)				
Substance name	**Average recovery**	**RSD [a]**	**n=**	**Spike range (mg/kg)**
Chlorpyrifos	83%	8.1%	4	0.005
Cypermethrin	116%	10%	6	0.005
Imidacloprid	117%	3.8%	6	0.005
PBO	64%	8.2%	6	0.001
Propoxur	94%	1.7%	6	0.001
Spinosad	78%	15%	4	0.001
Tebufenozide	71%	33%	6	0.001
Exp. 2 (+/-*MRL)				
Chlorpyrifos	77%	11%	4	0.005-0.025
Cypermethrin	105%*)	6.6%	2	0.005-0.025
Imidacloprid	98%	3.6%	4	0.005-0.025
PBO	94%	2.6%	2	0.005-0.025
Propoxur	91%	3.5%	4	0.005-0.025
Spinosad	98%	14%	3	0.005-0.025
Tebufenozide	120%	5.5%	4	0.005-0.025

*Legend: * Solvent only; a: Relative standard deviation*

Table S5. Overview of results for survival (n, number of larvae surviving) and increase in biomass (g) of black soldier fly larvae (Hermetia illucens) for Exp. 1 and Exp. 2. Mean and standard deviation.

Substance name(s)	Survival (n)		Increase in biomass (g)	
	Exp. 1 (1*MRL)	Exp. 2 (+/-*MRL)	Exp. 1 (1*MRL)	Exp. 2 (+/-*MRL)
Control (blank)	100.0 ± 0	-	11.1 ± 0.3	-
Control + MeOH	98.7 ± 0.6	99.0 ± 1.7	10.8 ± 0.3	11.0 ± 0.3
Control + ACN	99.7 ± 0.6	99.0 ± 1.0	10.8 ± 0.4	11.3 ± 0.5
Chlorpyrifos	99.3 ± 1.2	97.0 ± 2.6	10.9 ± 0.2	11.1 ± 1.0
Propoxur	97.3 ± 3.1	99.3 ± 0.6	10.6 ± 0.3	11.0 ± 0.1
Imidacloprid	99.3 ± 0.6	100.0 ± 0.0	13.0 ± 0.2	12.3 ± 0.6
Spinosad	38.3 ± 10.8	98.3 ± 2.9	3.4 ± 1.0	11.2 ± 0.3
Tebufenozide	99.3 ± 1.2	99.3 ± 0.6	10.9 ± 0.1	11.2 ± 0.6
Cypermethrin	87.3 ± 4.0	97.7 ± 3.2	8.2 ± 0.4	10.5 ± 0.1
Piperonyl butoxide	98.7 ± 1.2	99.7 ± 0.6	10.9 ± 0.0	11.8 ± 0.4
Cyperm. + PBO	77.7 ± 2.5	100.0 ± 0.0	4.5 ± 0.5	11.2 ± 0.7

Table S6: Analysed concentrations of the compounds in substrate, larvae and residual material (consisting of larval excreta + residual feed) in Exp. 1 (mg/kg). Mean and standard deviation (n = 3).

Substance name(s)	Analysed concentration in substrate (mg/kg)	Analysed concentration larvae (mg/kg)	Analysed concentration residual material (mg/kg)
Chlorpyrifos	0.03	POS (<0.005)	0.034 ± 0.004
Propoxur	0.05	<LOQ (0.001)	0.017 ± 0.007
Imidacloprid	0.1	POS (<0.005)	0.036 ± 0.005
Spinosad	1.4	0.122 [1]	1.731 ± 0.234
Tebufenozide	0.05	POS (<0.001)	0.020 ± 0.004
Cypermethrin	0.2	0.118 ± 0.018	0.927 ± 0.047
Piperonyl butoxide	6.3	0.026 ± 0.004	1.764 ± 0.136
Cypermethrin + Piperonyl butoxide	0.3 5.6	0.043 ± 0.008 0.065 ± 0.024	0.758 [1] 8.654 ± 0.724

<LOQ: below level of quantification (LOQ value indicated in brackets).
POS: positive value for the concentration, but could not be quantified (LOQ value indicated in brackets).
[1] Mean of two values.

Table S7: Analysed concentrations of compounds in larvae and residual material (consisting of larval excreta + residual feed) and spiked concentrations in substrate in Exp. 2 (mg/kg). Mean and standard deviation (n = 3).

Substance name(s)	**Spiked concentration in feed (mg/kg)**	**Analysed concentration larvae (mg/kg)**	**Analysed concentration residual material (mg/kg)**
Chlorpyrifos	0.5	0.012 ± 0.005	0.153 ±0.068
Propoxur	0.5	POS (<0.001)	0.036 ± 0.006
Imidacloprid	1.0	0.007 ± 0.001	0.446 ± 0.081
Spinosad	0.2	0.005 ± 0.002	0.099 ± 0.027
Tebufenozide	0.5	0.005 ± 0.001	0.066 ±0.010
Cypermethrin	0.1	0.079 ± 0.025	- [1]
Piperonyl butoxide	2.0	0.019 ± 0.007	0.375 ±0.050
Cypermethrin + Piperonyl butoxide	0.1 2.0	0.052 ± 0.019 0.021 ± 0.001	0.095 ± 0.020 0.428 ± 0.049

[1]: Due to a dilution error in sample preparation, the cypermethrin concentration in the excreta in Exp. 2 could not be quantified.
POS: positive value for the concentration but could not be quantified (LOQ value indicated in brackets).

Chapter 3

Effects of insecticides on lesser mealworm (*Alphitobius diaperinus*) – bioaccumulation, mortality, and growth

(Alphitobius diaperinus larvae. Photo by author.)

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Abstract

The lesser mealworm (LMW, *Alphitobius diaperinus* (Panzer); Coleoptera: Tenebrionidae) is increasingly used as a novel food and feed source. The feed materials on which this insect species is reared may contain residues of insecticides from agricultural use. The objective of this study was to determine whether selected insecticides, spiked to the substrate, could bioaccumulate in LMW and/or affect growth or survival. Insecticides were selected and spiked at concentrations equal to the legal limit in EU. Tested substances were chlorpyrifos, propoxur, imidacloprid, spinosad, tebufenozide, fipronil, pirimiphos methyl, cypermethrin, and the synergist piperonyl butoxide (PBO). Cypermethrin and PBO were also tested together. Insecticide concentrations in the spiked substrate and larvae were determined by LC-MS/MS. Concentrations in the larvae were largely below the limit of quantification (LOQ), meaning that bioaccumulation did not occur. Significant reductions in total yield were observed for spinosad (present in the substrate at 1.6 mg/kg) and imidacloprid (0.12 mg/kg). Spinosad is one of few insecticides that is permitted to be used in organic agriculture, which raises questions over the safety of organic produce for insect rearing. More research on the safety of organic produce treated with this insecticide for rearing of LMW is therefore highly recommended. The reported harmful effects of insecticide residues on LMW need to be accounted for by lowering the current legal limits or reconsidering approval for use. More research is advised particularly on sublethal effects of insecticide residues on LMW on adult beetles.

Introduction

Certain insect species are increasingly seen as an alternative protein source for food and feed (Van Huis et al., 2013). One such species is the lesser mealworm (LMW, *Alphitobius diaperinus* (Panzer); Coleoptera: Tenebrionidae) (Adámková et al., 2016; Janssen et al., 2017; Oonincx et al., 2020). This new type of livestock may be exposed to pesticide residues that can remain in the feed materials on which the insects are reared. Maximum residue limits (MRLs) for pesticides have been set in EU legislation at the lowest level achievable, "based on good agricultural practice and the lowest consumer exposure necessary to protect vulnerable consumers" (Regulation (EC) No 396/2009, Article 3.2(d)). In principle, MRLs laid down in Regulation (EC) No 396/2005 are applicable to both food and feed products (Article 18(1)). However, the effects of pesticide residues in plant-based materials in the context of rearing insects for food and feed have thus far not been accounted for.

Potential problems for insect farmers are twofold: firstly, the presence of insecticide residues in feed materials may reduce survival or induce a variety of adverse sub-lethal effects on growth, fecundity, and/or development (Desneux et al., 2007; Guedes et al., 2011). In a previous study, negative effects on the growth and survival of black soldier fly larvae (BSFL, *Hermetia illucens* (L.); Diptera: Stratiomyidae) were reported (Meijer et al., 2021). Since BSFL are phylogenetically unrelated to LMW, results may not be directly comparable and the need for more research on the effects of pesticide residues on more species of insects reared for food and feed is evident. Secondly, certain substances may bio-accumulate in the insects. Elevated concentrations could raise concerns on the safety of such products for consumers (Finke et al., 2015).

The focus of literature on the susceptibility of LMW to pesticides has thus far largely been on controlling this species as a pest in poultry farms. These experiments centred primarily on residual and topical contact toxicity (i.e. treated surfaces) (Despins et al., 1991; Hamm et al., 2006; Hickmann et al., 2018; Kaufman et al., 2008; Lambkin and Rice, 2007; Lyons et al., 2017; Oliveira et al., 2016; Salin et al., 2003; Steelman, 2008; Tomberlin et al., 2014; Vaughan and Turner Jr, 1984; Yeasmin et al., 2015; Yeasmin et al., 2014; Zafeiriadis et al., 2021). The predictive value of such data for field conditions is limited (Stark et al., 1995); which may also be the case for commercial rearing conditions – where exposure would be via consumption of homogenized feed. The number of studies that investigated the susceptibility of LMW to pesticides via the substrate appears to be limited, focusing on the category of insect growth regulators (IGRs) (Singh and Johnson, 2013; Zorzetti et al., 2015). Looking at taxonomically similar species, we identified one study that evaluated the susceptibility of adults, small and large larvae of yellow mealworm (YMW, *Tenebrio molitor* (L.): Coleoptera: Tenebrionidae) to four types of insecticides (pirimiphos-methyl, deltamethrin, spinosad and silicoSec) via contaminated feed, in the context of these substances being used as grain protectants (Kavallieratos et al., 2019). Authors reported YMW adults to be more susceptible to these insecticides than the larvae. In particular, pirimiphos-methyl

caused high mortality in small larvae and differences in effects were found depending on the type of grain the insects were reared on (barley, wheat, or maize).

The specific aims of this study were to determine the susceptibility of LMW to insecticide residues in the feed and to assess the potential bioaccumulation of these substances.

Methods and materials

In this study, LMW were exposed to a variety of insecticidal substances that had been mixed with the substrate on which the insects were reared. Eight different insecticides with different modes of action, and one synergist, were selected. Intended concentrations of these substances in the feed substrate were equal to the applicable MRL for maize. The LMW were reared on this spiked substrate for 14 days, since the younger larval instars are likely most susceptible to the effects of the insecticides. After the experiment, the larvae were killed by freezing. Susceptibility of the LMW to these substances was determined by comparing total yield, individual larval weight, and number of surviving larvae between treatments. Concentrations of insecticides in the larvae were analysed by LC-MS/MS. Bioaccumulation of tested substances was calculated, based on analysed concentrations in the larvae and substrate.

Selection of insecticidal substances

The insecticides investigated in this study – including the chemical class and mode of action (as defined by IRAC (Sparks et al., 2020) and applicable MRL for maize – are shown in Table 1. The selection of substances was based on our previous study on BSFL (Meijer et al., 2021), covering the following substances: chlorpyrifos, propoxur, imidacloprid, spinosad, tebufenozide, cypermethrin, piperonyl butoxide (PBO). Imidacloprid as an active substance in plant protection products was still permitted to be used as a seed coating for crops grown in permanent greenhouses at the time that the experiments were conducted (Regulation (EU) No 2018/783). Although the EU-wide approval has expired since then (Regulation (EU) 2020/1643), individual EU member states have authorized imidacloprid for various uses, using emergency derogation powers (Article 53 of Regulation (EC) No 1107/2009).

In addition to the selection of insecticides in Meijer et al. (2021), the phenylpyrazole fipronil and organophosphate pirimiphos-methyl were also tested in this study. Pirimiphos-methyl was added because of the high mortality found in the taxonomically related YMW (Kavallieratos et al., 2019). Fipronil was included to test an additional mode of action: GABA-gated chloride channel (GGCC) blockers (Simon-Delso et al., 2015; Sparks et al., 2020).

Table 1: Selected insecticides including class, mode of action, and maximum residue level (MRL) in the EU for maize (Regulation (EC) No 396/2005).

Substance name	**Class**	**Mode of Action**	**MRL (mg/kg)**
Chlorpyrifos	Organophosphates	Acetylcholinesterase (AChE) inhibitors	0.05
Propoxur	Carbamates	Acetylcholinesterase (AChE) inhibitors	0.05*
Imidacloprid	Neonicotinoids	Nicotinic acetylcholine receptor (NAchR) competitive modulators	0.1
Spinosad	Spinosyns	Nicotinic acetylcholine receptor (NAchR) allosteric modulators – site I	2.0
Tebufenozide	Insect growth regulators (IGRs)	Ecdysone receptor agonists	0.05*
Fipronil	Phenylpyrazoles (fiproles)	GABA-gated chloride channel blockers	0.005*
Pirimiphos methyl	Organophosphates	Acetylcholinesterase (AChE) inhibitors	0.5
Cypermethrin	Pyrethroids	Sodium channel modulators	0.3
Piperonyl butoxide (PBO)	Synergist	Synergist	No MRL

**: Indicates lower limit of analytical determination.*

The effects of selected insecticides on LMW were tested in two sequential experiments. In the first experiment ('Exp. 1'), the substances chlorpyrifos, propoxur, imidacloprid, spinosad, tebufenozide, fipronil, and pirimiphos methyl were tested. This experiment was performed with each treatment in triplicate. In the second experiment ('Exp. 2'), the effects of cypermethrin and PBO were tested with 5 replicates per treatment. Exp. 2 was held at a smaller scale than Exp. 1. The larger number of replicates in Exp. 2 (n = 5) were used to be able to determine whether the presumed synergistic effects of PBO were statistically significant, by comparing the results of the treatment containing both cypermethrin and PBO to the treatment containing only cypermethrin. The other substances tested in Exp. 1 were compared against the (pooled, n = 9) controls, for which n = 3 per treatment were considered to suffice.

Feed preparation

A test feed ('substrate') was composed for both experiments, consisting of a dry mix of primarily organic wheat products, a vegetable protein source, and a pre-mix (Research Diet Services, Wijk bij Duurstede, The Netherlands). This substrate was provided by Protifarm B.V. (Ermelo, The Netherlands), where the experiments took place. The substrate was prepared for the experiments at the Wageningen Food Safety Research (WFSR) laboratory by spiking with insecticides dissolved in either acetonitril (ACN) or methanol (MeOH). The intended concentration of each insecticide was equal to the selected MRL (Table 1). Since PBO is not a plant protection product, it does not have an MRL. Therefore, it was used at a ratio of 1:20 (cypermethrin:PBO), which is a ratio used in commercial formulations for application of this pyrethroid / synergist combination in the field (Osimitz, 2010), similar to Meijer et al. (2021). The list of

treatments, spiked concentrations, solvent, purity (%), and suppliers is provided in Table 2.

Table 2: Insecticides tested, their intended spiked concentration in the feed, solvent, purity, and suppliers.

Substance	**Spiked concentration (mg/kg)**	**Solvent**	**Purity (%)**	**Supplier**
Chlorpyrifos	0.05	MeOH	99.3	Sigma-Aldrich[1]
Propoxur	0.05	MeOH	99.9	HPC[2]
Imidacloprid	0.1	MeOH	98.7	HPC[2]
Spinosad	2.0	MeOH	96.6	HPC[2]
Tebufenozide	0.05	ACN	99.9	Sigma-Aldrich[1]
Fipronil	0.005	MeOH	97.3	HPC[2]
Pirimiphos-methyl	0.5	ACN	98.5	Sigma-Aldrich[1]
Cypermethrin	0.3	ACN	99.7	HPC[2]
Piperonyl butoxide (PBO)	6.0	ACN	92.5	Dr. Ehrenstorfer[3]
Cypermethrin + PBO	0.3 + 6.0	ACN	99.7 + 92.5	HPC[2] + Dr. Ehrenstorfer[3]

1: Sigma-Aldrich Chemie N.V., Postbus 27, 3330 AA Zwijndrecht, The Netherlands
2: HPC Standards GmbH, Am Wieseneck 7, 04451 Cunnersdorf, Germany
3: LGC Standards GmbH, Mercatorstrasse 51, 46485 Wesel, Germany

Based on practical experiences, 0.275 kg of feed would be required per replicate for Exp. 1 (chlorpyrifos, propoxur, imidacloprid, spinosad, tebufenozide, fipronil, and pirimiphos methyl). Therefore, per treatment at least 0.825 kg of feed (3 x 0.275) was required to be spiked. In order to mix the insecticides homogenously with the provided dry feed, tap water was added before the spiking and subsequent mixing. Per treatment, 1 kg of feed was mixed with 3 l water, using a UM 12 table-top mixer (Stephan Machinery GmbH, Hameln, Germany) at 1500 rpm for 2 min. The intended concentration, and therefore the total volume of insecticides and respective solvents, differed per treatment. In order for the volume of solvents to be equal in all treatments, additional MeOH or ACN was added: bringing the total volume to 1.0 ml (MeOH) and 3.5 ml (ACN) per treatment, respectively. The same volumes were also added to the two respective solvent controls. The resulting slurry was moved into 3 plastic zipper bags each per treatment, which were put into crates, and were then frozen at – 18°C. The frozen spiked substrate was freeze-dried in batches in a pilot scale tray freeze dryer (Scala Scientific, Ede, The Netherlands) at Unifarm (Wageningen University & Research, Wageningen, The Netherlands) until the shelf temperature was approximately equal to the product temperature.

In Exp. 2, the following four treatments were tested: only cypermethrin, only PBO, both cypermethrin and PBO, and a blank control. It was decided to only include a blank control (and no solvent control) in Exp. 2 because the differences of solvent controls with the blank control in Exp. 1 showed not to be significant ($P > 0.05$). The feed preparation procedure in Exp. 2 was the same as in Exp. 1, except that a) 75 g of feed was used per replicate, and b) the spiked feed was freeze-dried using a Millrock REVO

Series Freeze-Dryer Model LD 85 (Millrock Technology, Kingston, USA) at WFSR (Wageningen, The Netherlands).

Experimental procedures

For Exp. 1, per replicate, 1.85 g of first instars LMW, originating from one cohort of eggs, were weighed and put in a small container (19 x 11.5 cm, Dampack International, Werkendam, The Netherlands). The containers were prepared with a small amount of dry meal and a small amount of wetted meal (water to meal ratio 2:1), to provide moisture. During the 14-day rearing period, additional wetted meal was provided daily – amounts corresponding to the growth of the larvae. Over the 14-day rearing period, 275 g of meal was provided in total per replicate. During feeding, for every treatment, clean gloves were used to avoid cross-contamination of the different treatments. After 14 days, the larvae were separated from the feed substrate residue by sieving. The insects were thoroughly cleaned by shaking the insects over a 1 mm sieve so all attached faecal and feed particles were removed from the animals. The insects were weighed to determine total yield (g) per replicate. For each replicate, a representative sample (600-800 mg), accurately weighed, was taken. The number of larvae in this sample was counted twice to calculate the average individual weight of the larvae (mg). This individual larval weight was used to calculate the total number of larvae in the replicate (based on total weight of the replicate). Finally, insects were killed by freezing at -18 °C.

The animal procedures of Exp. 2 were identical to Exp. 1, with the exception that 0.4 g of first instar LMW were reared per replicate, and a smaller container (11.5 x 11.5 cm, Dampack International, Werkendam, The Netherlands) was used - both to account for the reduced amount of substrate that was provided in Exp. 2.

Chemical analyses and quality control

Analyses were performed using the method applied in Meijer et al. (2021). In brief, the active compounds were extracted from the frozen larval samples (1.0 g (± 0.05 g)) and analysed by liquid chromatography-mass spectrometry (LC/MS-MS). UPLC gradient conditions for all substances are listed in Table S1. MS/MS conditions of the compounds fipronil and pirimiphos-methyl, which were additional to the mentioned previous study, are presented in Table S2.

Quality control (QC) was performed by spiking blank samples with active substances in ranges corresponding to the spiked concentration, see Table S3.

Calculations and statistical analyses

The bioaccumulation factor of tested substances was calculated by dividing the analysed concentration in the larvae by the concentration in the substrate, applying the procedure used in Van der Fels-Klerx et al. (2016).

Statistical analyses were performed using the software SPSS Statistics for Microsoft Windows (version 25.0.0.2, IBM Corp., Armonk, NY, United States). Non-parametric

statistical tests were used because the number of replicates per treatment did not warrant tests on conformity to a distribution type. Statistically tested variables were total yield (g), individual larval weight (mg), and number of larvae (n). For Exp. 1 (n = 3), differences in each of these three variables between the blank and two solvent controls were analysed using Kruskal-Wallis tests (α = 0.05). If differences were not significant for the three controls (P > 0.05), the controls were pooled (i.e. n = 9) to compare the values of the controls with those of the spiked treatments (Kruskal-Wallis tests (α = 0.05)). For variables for which differences were significant (P < 0.05), results for each spiked treatment were compared to the pooled controls using a Mann-Whitney U test. Because this post-hoc test was a multiple comparison test, a lower significance level of 0.01 was used.

The same statistical tests were used for Exp. 2, but the treatments were compared against the blank control (n = 5) that was used in that experiment (Kruskal-Wallis, α = 0.05). For variables for which differences were significant (P < 0.05), results for each spiked treatment were compared to the control using a Mann-Whitney U test (α = 0.01). Finally, for each of the variables, differences between the treatment containing cypermethrin were compared to the treatment that contained both cypermethrin and PBO, using a Mann-Whitney U test (α = 0.01).

Results

Quality control

The results of the quality control analyses of the substrate are shown in Table S3. For all treatments, except for pirimiphos-methyl, the mean recovery was within the acceptable range of 70-120% (SANTE, 2019). For pirimiphos-methyl, recovery was 65.1%. Recovery correction is acceptable when the recovery is outside of the range of 80-120% (but within 30-140%), and in that case the recovery should be multiplied with a factor [100 %/recovery %] (SANTE, 2019). The measured concentration for pirimiphos-methyl (of 0.42 mg/kg) was therefore corrected to 0.64 mg/kg.

The results of the quality control analyses of the larvae are shown in Table S4. The average recovery of fipronil in the larval samples was insufficient. Hence, we do not present concentrations in the larvae and the bioaccumulation factor of fipronil.

Larval survival and growth

Results for the variables total yield, individual larval weight, and number of larvae (combined for both experiments) are shown in Table S5. For number of larvae, yield and individual larval weight; differences were not significant between the blank and two solvent controls (P > 0.05) in Exp. 1. Therefore, the three controls (n = 9) were pooled for further statistical analysis.

In Exp. 1, differences in total yield (P = 0.05) and individual larval weight (P = 0.046) were significant, but the number of larvae was not affected (P = 0.267). The post-hoc tests showed a significant reduction in total yield by imidacloprid (P = 0.009) and

spinosad (P = 0.009). The post-hoc tests for individual larval weight showed no significant differences between each of the spiked treatments and the pooled controls (P > 0.01).

In Exp. 2, differences were significant for total yield (P = 0.03) and individual larval weight (P = 0.024) but, similar to Exp. 1, the number of larvae did not differ (P = 0.268). The post-hoc tests for total yield and individual larval weight showed no significant differences between any of the treatments and the control (P > 0.01). However, differences between the treatment containing only cypermethrin and the treatment containing both cypermethrin and PBO were significant, both for total yield (P = 0.008) and individual larval weight (P = 0.008).

Results for total yield and individual larval weight are shown in Figure 1 and Figure 2, respectively, combined for both experiments. The values in the boxplots for each of the treatments are expressed as a percentage of the mean control values in the respective experiment. For Exp. 1 (chlorpyrifos, propoxur, imidacloprid, spinosad, tebufenozide, fipronil, and pirimiphos-methyl), this was the mean of the pooled controls, whereas this was the blank control for Exp. 2 (cypermethrin and PBO).

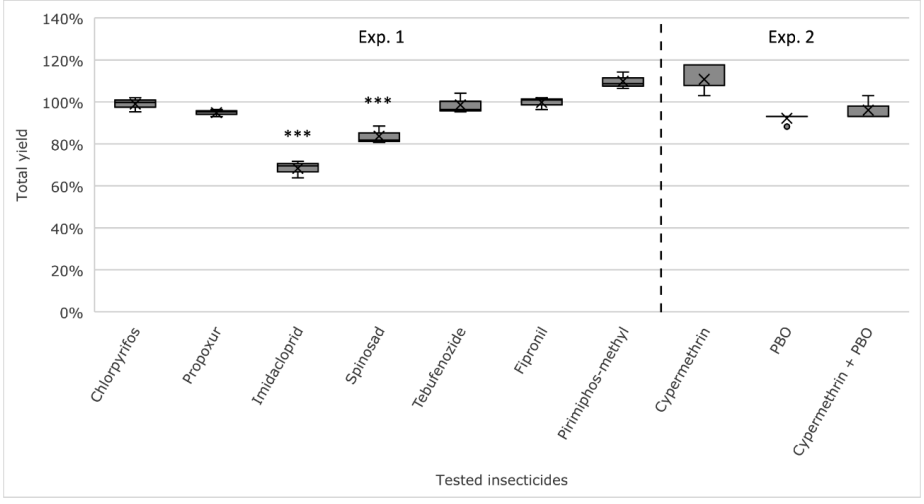

*Figure 1: Percentage of total larval yield for each of the treatments (box plots). For Exp. 1 (chlorpyrifos, propoxur, imidacloprid, spinosad, tebufenozide, fipronil, and pirimiphos-methyl), this is expressed as the percentage of the treatment (n = 3) relative to the mean of the pooled values for the blank and two solvent controls (n = 9). For Exp. 2 (cypermethrin and piperonyl butoxide (PBO) (and the treatment combining both)), the mean of the blank control (Exp. 2; n = 5) was used as the reference in the calculation of the percentage. Significance of differences between a treatment and the respective control(s) (P < 0.01) is denoted by ***.*

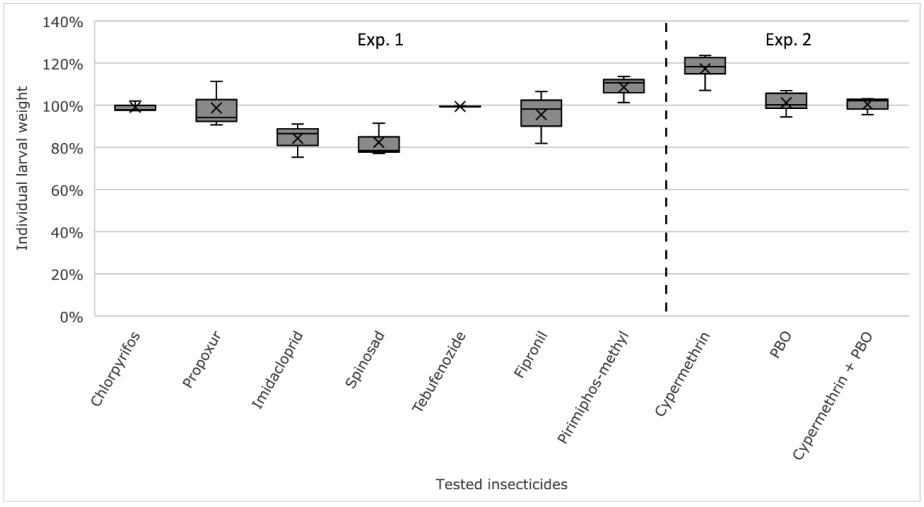

Figure 2: Percentage of individual larval weight for each of the treatments (box plots). For Exp. 1 (chlorpyrifos, propoxur, imidacloprid, spinosad, tebufenozide, fipronil, and pirimiphos-methyl), this is expressed as the percentage of the treatment (n = 3) relative to the mean of the pooled values for the blank and two solvent controls (n = 9). For Exp. 2 (cypermethrin and piperonyl butoxide (PBO) (and the treatment combining both)), the mean of the blank control (Exp. 2; n = 5) was used as the reference in the calculation of the percentage.

Concentrations and bioaccumulation

Table 3 presents the concentrations of the substances in the larvae. For almost all tested substances, concentrations in the larvae were below their respective limit of quantification (< LOQ), except for spinosad, which had a very low concentration in the larvae. Given the insecticide being absent or very low in the larvae, accurate bio-accumulation factors could, not be calculated.

Table 3: Concentrations (mg/kg) of tested insecticides in spiked feed (pre-experiment) and larvae (post-experiment) as quantified by LC-MS.

Substance	**Concentration feed (mg/kg)**	**Concentration larvae (mg/kg)**
Chlorpyrifos	0.04	< LOQ (0.005)
Propoxur	0.04	< LOQ (0.001)
Imidacloprid	0.12	< LOQ (0.001)
Spinosad	1.58	0.001
Tebufenozide	0.05	< LOQ (0.005)
Fipronil	0.01	n/a *
Pirimiphos methyl	0.64 **	< LOQ (0.001)
Cypermethrin	0.38	< LOQ (0.025)
Piperonyl butoxide (PBO)	5.1	< LOQ (0.025)
Cypermethrin + PBO	0.28 5.1	< LOQ (0.025) < LOQ (0.025)

**: The analytical results for the concentration of fipronil in larval samples are not presented because the recovery of fipronil in the quality control analyses was insufficient.*
***: Because the recovery of pirimiphos-methyl in quality control checks was < 70% (see Table S3), the analysed concentration was corrected.*

Discussion

Five out of eight insecticides tested in this study (chlorpyrifos, propoxur, tebufenozide, fipronil, and pirimiphos-methyl) did not significantly affect total yield, individual larval weight, and number of surviving larvae of LMW. However, the absence of significant effects in this experiment does not exclude the possibility of adverse effects in life-stages other than the larvae. Differences in susceptibility to pesticides - depending on the life stage of an insect (adult/larvae) (Hickmann et al., 2018; Zafeiriadis et al., 2021) and manner of application (topical / residual) (Steelman, 2008) - have been observed for LMW and other Tenebrionidae (Kavallieratos et al., 2019). Furthermore, it is also possible that exposure to (sub-) lethal concentrations of pesticides in the diet manifest as adverse effects in a later life-stage; e.g. by affecting pupation, adult emergence, or fecundity (De França et al., 2017; Desneux et al., 2007; Haynes, 1988). More research is therefore required to determine the susceptibility of other LMW life-stages when exposed to insecticide residues in the substrate.

The advice to study the effects of insecticides on other LMW life stages is especially pertinent for insect growth regulators (IGRs), such as tebufenozide tested in this study. Tebufenozide is a synthetic ecdysone agonist that interferes with the moulting process, thereby halting development (Barnett and Brundage, 2010). Tebufenozide is most effective against Lepidoptera, but less so against Coleoptera (Nakagawa, 2005). Tebufenozide was initially selected due to the presence of residues found in feed materials, reported in RASFF (Meijer et al., 2021).

A significant reduction in total yield was observed in the two treatments containing imidacloprid (68.4 ± 4.0% of mean value of pooled controls) and spinosad (83.7 ± 4.2%). The susceptibility of LMW to both substances is well studied in terms of topical and residual toxicity (Singh and Johnson, 2015; Yeasmin et al., 2015; Zafeiriadis et al., 2021). Both compounds target the nicotinic acetylcholine receptor (nAChR) (Casida, 2009; Sparks et al., 2020). The highest concentration in this study (2.0 mg/kg) was used for spinosad, and this high dose is thought to play a role in the observed effects. A concentration in the range of 0.005 - 0.5 mg/kg, like the other tested substances, would be expected to cause substantially weaker effects. Notably, spinosad is one of the few insecticides that is permitted to be used as a plant protection product in organic farming in the EU (Regulation (EC) No 889/2008, Annex II). This raises questions over the presumed safety of organic produce for LMW rearing if spinosad residues persist in the feed on which the insects are reared. Imidacloprid caused a more severe reduction in total yield, despite the lower concentration used (0.1 mg/kg), compared to spinosad's (2.0 mg/kg). Although the EU-wide approval of imidacloprid has expired, it is still permitted to be used in several countries by derogation, and feed materials may therefore continue to contain residues of imidacloprid at low concentrations. The results of this study suggest that such concentrations can cause mortality in LMW.

Differences in total yield and individual larval weight were significant between the treatment containing only cypermethrin, and the treatment containing both

cypermethrin and PBO, but not between either of these treatments and the control. Cypermethrin appeared to cause a slight increase in total yield as compared to the control (110.8 ± 6.6 %), while the addition of PBO caused a minor decrease (96.1 ± 4.4 %). We could not identify any published study on hormesis in Coleoptera by cypermethrin. It is, therefore, unclear what could have caused this increase in total yield.

The concentrations of almost all tested substances – except spinosad, which was very low (0.001 mg/kg) – in the larvae were below the limit of quantification. Concerns over bioaccumulation of these substances can, therefore, be ruled out and the presence of residues of these insecticides at the concentrations in the substrate tested is not likely to pose a safety risk for the animal or human that will be consuming the final insect product.

The concentration of fipronil in the larvae is not reported because recovery of this substance in the quality control analyses was insufficient. It is unclear what may have caused this. One possible explanation could be interference in the analysis by the larval matrix.

It must be highlighted that the results of this study may be limited to the particular LMW strain that was used in these experiments. Several studies have found significant inter-population variation in susceptibility of LMW, collected from poultry farms, to a variety of pesticides, including the substances imidacloprid, spinosad, and cypermethrin which significantly affected LMW larvae in this study (Hickmann et al., 2018; Renault and Colinet, 2021; Singh and Johnson, 2015). Resistance to the pesticides reported in the latter studies correlated to long-term exposure of populations to sub-lethal doses. The LMW strain used in this study has been reared in captivity for over 35 years. Chronic exposure to sub-lethal insecticide concentrations in that time is possible. Therefore, the particular strain that was used for these experiments may have developed resistance to the tested substances, that may not be present in other populations. It is therefore advisable to verify these results in other commercial LMW strains.

Conclusion and recommendations

The aims of this study were to determine the susceptibility of LMW to insecticide residues in the feed and to assess the potential bioaccumulation of these substances. This was done for the active substances chlorpyrifos, propoxur, imidacloprid, spinosad, tebufenozide, fipronil, pirimiphos-methyl, and cypermethrin. The latter substance was also tested in combination with the synergist PBO. We conclude that growth and survival of LMW may be negatively affected by spinosad and imidacloprid at insecticide residue concentrations that are currently allowed in animal feed in the EU. Cypermethrin exposure resulted into an increase in LMW total yield and individual larval weight, but the addition of PBO negated this effect. All concentrations of the tested substances in the larvae were <LOQ and bioaccumulation must thus be assumed to be nil, given the insecticide concentrations around MRL levels in the substrate. LMW products from

insects exposed to residues of tested insecticides during rearing at concentrations equal to the MRL can therefore be considered safe for consumers.

Based on our results, we strongly recommend policymakers to consider adopting insect species-specific MRLs for feed materials. The effects of insecticides on commercial insect rearing for food and feed should also be taken into account for the approval and legal limits of insecticides, as is already being done for beneficial insects such as pollinators. More research should be done on the safety for insects reared on organic feed treated with spinosad. Insect farmers are advised to be diligent in analysing incoming feed materials for the presence of insecticide residues, as this may significantly affect total yield. More research is needed on a variety of insecticidal substances. In addition, it is encouraged to assess potential sub-lethal effects of insecticides on other life-stages of the insects than the larval stage, in particular the adult stage essential for reproduction, and using other commercial insect strains.

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Supplementary materials

Table S1. UPLC Gradient conditions for all tested substances.

Time (min)	Flow (ml/min)	A%	B%
-	0.4	100	0
1	0.4	100	0
6	0.4	0	100
9	0.4	0	100
9.5	0.4	100	0
12	0.4	100	0

Table S2. MS/MS conditions for the substances fipronil and pirimiphos-methyl.

Q1	Q3	Substance ID	DP	CE	CXP
434.9	434.9	Fipronil	-50	-18	-8
434.9	434.9		-50	-36	-8
450.9	450.9	Fipronil-sulfone	-30	-34	-8
450.9	450.9		-30	-20	-8
306.1	306.1	Pirimiphos-methyl	46	29	20
306.1	306.1		46	39	20

Legend: Q1: first quadrupole; Q3: third quadrupole; DP: declustering potential; CE: collision energy; CXP: cell exit potential

Table S3. Quality control results analytical procedure (substrate), for all tested substances.

Substance	Intended concentration (mg/kg)	Average recovery	RSD [a]	N	Spike range (ng/g)	Analysed concentration (mg/kg)
Chlorpyrifos	0.05	95.2%	-	-	100-200	0.04
Propoxur	0.05	105.4%	-	-	5-50	0.04
Imidacloprid	0.1	90.4%	-	-	5-50	0.12
Spinosad	2.0	103.8%	-	-	5-100	1.58
Tebufenozide	0.05	84.3%	-	-	50	0.05
Fipronil	0.005	77.1%	-	-	5-50	0.01
Pirimiphos methyl	0.5	65.1%	-	-	5-50	0.64 *
Cypermethrin	0.3	80.7%	6.2	6	50	0.38
Piperonyl butoxide (PBO)	6.0	75.6%	8.8	6	5-50	5.1
Cypermethrin + PBO	0.3 6.0	80.7%	2.9 3.3	6	50	0.28 5.1

[a]: Relative standard deviation.
**: because the recovery of pirimiphos-methyl was < 70%, the analysed concentration was corrected.*

Table S4. Quality control results analytical procedure (larvae), for all tested substances.

Substance	Average recovery	RSD [a]	N	Spike range (ng/g)
Chlorpyrifos	99% 104%	13% 8.7%	3 3	5 25
Propoxur	95% 121%	6.1% 4.7%	3 3	5 25
Imidacloprid	122% 123%	0.4% 8.3%	2 2	5 25
Spinosad	94% 96%	8% 5.6%	3 3	5 25
Tebufenozide	138% 97%	20% 20%	3 3	5 25
Fipronil	48% 40%	12% 19%	3 3	5 25
Fipronil-sulfon	91% 97%	8% 6%	3 3	5 25
Pirimiphos methyl	100% 103%	6.3% 4.8%	3 3	5 25
Cypermethrin	- 74%	- -	- 1	5 25
Piperonyl butoxide (PBO)	67%	6.5% 24%	2 3	5 25
Cypermethrin PBO	- 74% 67% 83%	- - 6.5% 24%	- 1 2 3	5 25 5 25

[a]: *Relative standard deviation.*

Table S5. Total yield (g), individual larval weight (mg), and number of larvae (n) per treatment. Mean and standard deviation (SD).

Tested insecticides	Total yield (g)	Individual larval weight (mg)	Number of larvae (n)
Experiment 1 (Chlorpyrifos, Propoxur, Imidacloprid, Spinosad, Tebufenozide, Fipronil, Pirimiphos methyl)			
Chlorpyrifos	88.3 ± 3.1	4.29 ± 0.11	20602 ± 772
Propoxur	84.7 ± 1.5	4.27 ± 0.48	19961 ± 2017
Imidacloprid	61.0 ± 3.6 ***	3.65 ± 0.35	16761 ± 919
Spinosad	74.7 ± 3.8 ***	3.57 ± 0.34	21082 ± 2430
Tebufenozide	88.0 ± 4.4	4.30 ± 0.01	20441 ± 957
Fipronil	89.0 ± 2.6	4.14 ± 0.54	21784 ± 3132
Pirimiphos methyl	98.0 ± 3.6	4.70 ± 0.28	20890 ± 1031
Control (ACN)	93.0 ± 2.6	4.56 ± 0.38	20452 ± 1211
Control (MeOH)	85.3 ± 5.7	4.06 ± 0.17	21015 ± 1025
Control (blank)	89.3 ± 5.9	4.36 ± 0.45	20728 ± 3642
Experiment 2 (cypermethrin + PBO)			
Cypermethrin	22.6 ± 1.3	4.64 ± 0.26	4878 ± 357
Piperonyl butoxide (PBO)	18.8 ± 0.4	4.01 ± 0.21	4706 ± 321
Cypermethrin + PBO	19.6 ± 0.9	3.97 ± 0.13	4938 ± 300
Control (blank)	20.4 ± 1.1	3.96 ± 0.48	5189 ± 367

***: *Significant difference to the mean of the pooled controls ($P < 0.01$).*

Chapter 4

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

(Hermetia illucens adult fly. Photo by author.)

This chapter is based on: Meijer, N., Zoet, L. de Rijk, T. Zomer, P., Rijkers, D., Van der Fels-Klerx, H.J., Van Loon, J.J.A. (2023). Effects of pyrethroid and organophosphate insecticides on reared black soldier fly larvae (*Hermetia illucens*). *Insect Science*. https://doi.org/10.1111/1744-7917.13269

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 feed materials on which they are reared. This study aimed to investigate the effects of six different insecticides belonging to the pyrethroid and organophosphate classes on the performance of this insect species. 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 for pirimiphos-methyl (4.8 mg/kg) were determined. The addition of PBO to cypermethrin enhanced its relative potency with a factor 2.6. At a cypermethrin to PBO ratio of 1:20, the CED10 was 0.2 mg/kg. 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. Negative effects were also observed for exposure to multiple substances at lower concentrations, suggesting additive and synergistic effects. Of the tested substances, deltamethrin was most toxic, causing 94% yield reduction even at 0.5 mg/kg. Analytical results suggest 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 non-compliance. It is recommended that rearing companies implement lower contractual thresholds, and that policymakers consider adjusting legally allowed maximum residue levels in insect feed.

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 and 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 sub-lethal effects (Guedes et al., 2011, Desneux et al., 2007) 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 insect-derived products (Meyer et al., 2021, EFSA, 2015). In a previous study, a significant increase in mortality and reduction in total yield was observed when 7-day 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 days (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 non-compliance 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 (Poma et al., 2022, Labu 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 (Geissen et al., 2021, EFSA, 2020). 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 (Hirano, 1989, Elliott et al., 1978, Siegfried and Scharf, 2001). However, results of such studies may not necessarily translate well to the conditions of commercial mass-rearing settings of non-target 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 (Soderlund, 2010, Wakeling et al., 2012, Khambay and Jewess, 2004), but often recovery is observed after a few days in laboratory settings, even at high doses (Khambay and Jewess, 2004). 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 (Khambay and Jewess, 2004, Casida et al., 1983). 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 and Jewess, 2004). 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 (Khambay and Jewess, 2004, Bhatt et al., 2020, Bradbury and Coats, 1989, Scott, 2001).

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 (Romero et al., 2009, Young et al., 2005, 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 and Scharf, 2001). The toxicity of some organophosphates is dependent on oxidative bio-activation by P450 monooxygenases (Siegfried and Scharf, 2001). Detoxification and metabolization of organophosphates has been linked to P450 monooxygenases, glutathione S-transferases, and hydrolytic enzymes (Siegfried and 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 (Yunta et al., 2019, Scott, 1999, Kolaczinski and Curtis, 2004, Cilek et al., 1995, Smith 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 bio-activation (dichlorvos) was unaffected by the addition of PBO (Ankley and Collyard, 1995). As such, we hypothesized that the addition of PBO to a P450 bio-activated 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-day old *Hermetia illucens* (BSF) larvae were exposed to pyrethroids, organophosphates and combinations in the feed substrate for a period of 7 days. 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-day old BSFL, was also conducted. The methodology and results of this separate experiment are presented in Appendix D.

Selection of treatments

The objective of Exp. 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 Exp. 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 ½ 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.

Exp. 2 aimed to gather more data needed to construct concentration/response (C/R) curves for the effects of tested substances on BSF yield. The choices of treatments in Exp. 2 were partly based on the results of Exp. 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.

Exp. 3 aimed to assess the effects 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 Exp. 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 Exp. 3 for the model substances, as well as for the four selected analogues. Two treatments 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 yield. 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 Exp. 3 were analysed to determine concentrations in this matrix, and assess transfer from the substrate to the larvae. Analytical details are provided in section 2.5 below.

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

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

Treatment	Substance(s) per treatment	Exp. 1	Exp. 2	Exp. 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: non-applicable.

Substrate preparation

Details of the used insecticide reference standards are shown in Table A.1. 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 Mueller-Maatsch (Submitted). 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 centre 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-day 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 mould 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 sub-lethality, 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 Exp. 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, United States). Non-parametric 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 Exp. 1 and Exp. 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 section 2.1.

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 analysed 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*exp(bx^d) with a > 0, d > 0) and E5-CED (y=a*[c-(c-1)exp(-bx^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* equalling infinity. Biologically, the curve levelling 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, i.e., 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 Exp. 1 and 2 compared to Exp. 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, i.e., 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 5^{th} and 95^{th} percentiles of the bootstrap runs in brackets.

Chemical analyses

Feed samples from all treatments were analysed 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 Exp. 1 was verified by analysing 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 Exp. 3 were analysed 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 analysed concentrations, carry-over, 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.

Monitoring data

For this study, ForFarmers Corporate Services BV provided data on the presence of insecticides in feed materials, gathered via their yearly monitoring programme of animal feed products during the 2020 and 2021 harvests. Samples were analysed by EN-ISO17025:2017 accredited external laboratory Primoris (Belac Accreditation number: 057), using a multi-residue-method employing GC-MS/MS and LC-MS/MS. These monitoring data of insecticide residues in commercial animal feed are shown in Table C.1. in Appendix C. In total 90 samples were analysed 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 analysed 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 analysed concentrations, and observed survival (%), biomass yield (g), and mean individual larval weight (mg) results are shown in Table B.1 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 (Figure 1). The AIC value of this model was -50.48. The software classified the yield for the treatment with analysed 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 analysed 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.

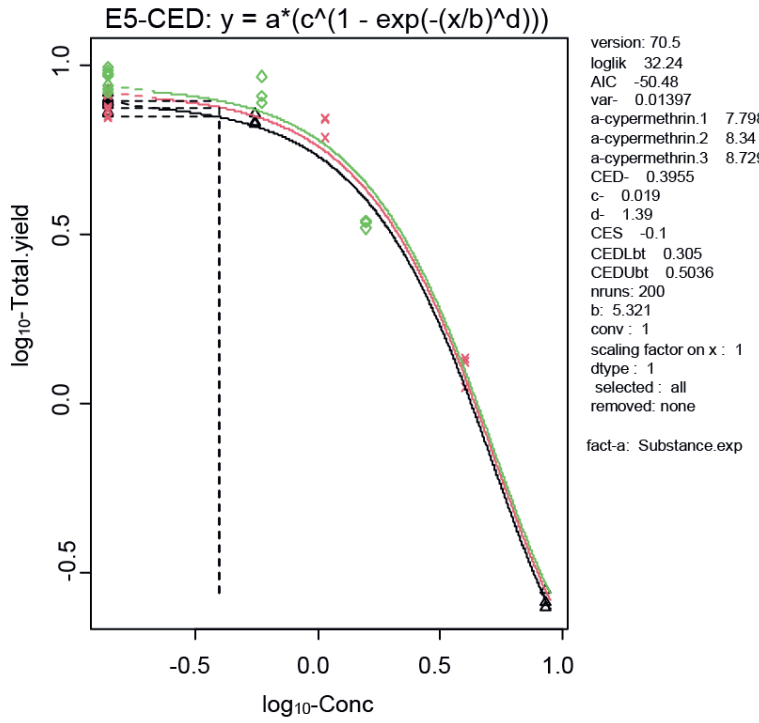

Figure 1: Concentration/response curves for the effect of the concentration (mg/kg) of cypermethrin (CYP) on total black soldier fly larvae (BSFL) biomass yield (g) on log_{10}-scale axes. The colours in the plot relate to the following subgroups (experiments): black/triangle (Exp. 1), red/cross (Exp. 2), green/diamond (Exp. 3).

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 (Figure 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, therefore, did not affect yield. The monitoring data shown in Table C.1 and C.2 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.

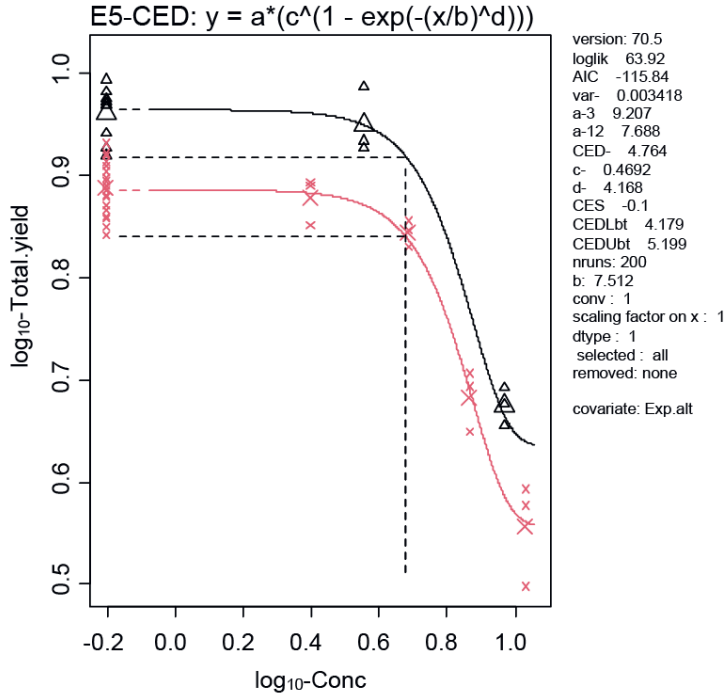

Figure 2: Concentration/response curves for the effect of the concentration (mg/kg) of pirimiphos-methyl (PM) on the total black soldier fly larvae (BSFL) biomass yield (g) on log_{10}-scale axes. The colours in the plot relate to the following subgroups (experiments): red/cross (Exp. 1 and 2); black/triangle (Exp. 3).

Piperonyl butoxide (PBO) synergism

The results for all treatments containing both CYP and PBO (PBO) at different ratios are shown in Figure 3. CYP and PBO were tested at ratios of 1:1, 1:10, and 1:20 in Exp. 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 Exp. 2 and 3, in order to gather more data for a C/R curve.

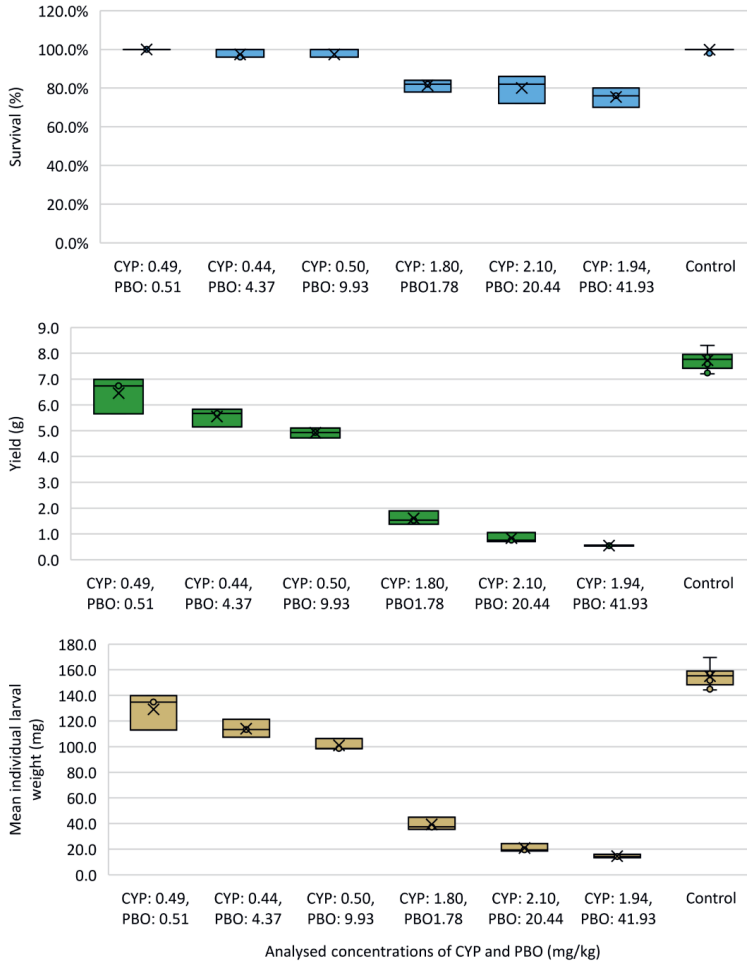

Figure 3: Survival (%), yield (g) and mean individual larval weight (mg) in response to exposure to both cypermethrin (CYP) and piperonyl butoxide (PBO), for the concentrations analysed (mg/kg). Box plots show median (-) and quartiles; X = arithmetic mean for n = 3 replicates per treatment, and n=9 for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

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. Firstly, the results were plotted by considering only the CYP concentration in these treatments, i.e. 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 (Figure 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 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 %).

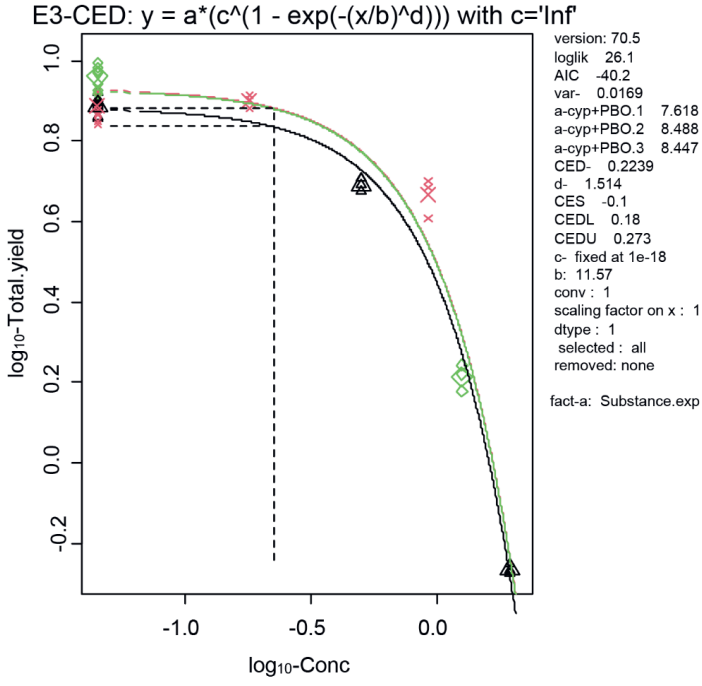

Figure 4: Concentration/response curves for the effect of the concentration (mg/kg) of cypermethrin (CYP) in combination with piperonyl butoxide (PBO) on total black soldier fly larvae (BSFL) biomass yield (g) on log_{10}-scale axes. The colours in the plot relate to the following subgroups (experiments): black/triangle (Exp. 1), red/cross (Exp. 2), green/diamond (Exp. 3).

Results of the modelling analysis on the relative potency of PBO when used in conjunction with CYP are shown in Figure 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.

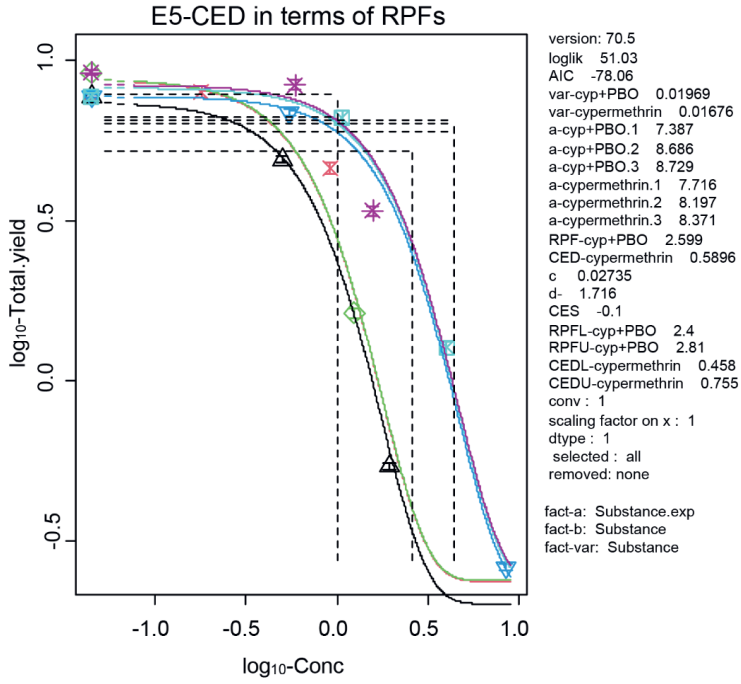

Figure 5: Concentration/response curve for the relative potency factor (RPF) of the effect of the concentration (mg/kg) on total black soldier fly larvae (BSFL) biomass yield (g), for cypermethrin (CYP) combined with piperonyl butoxide (PBO) against CYP alone on log$_{10}$-scale axes. The colours in the plot relate to the following subgroups (experiments): black/upward triangle (CYP+PBO, experiment 1), red/cross (CYP+PBO, exp. 2), green/diamond (CYP+PBO, exp. 3); dark blue/downward triangle (CYP, Exp. 1), light blue/cross-square (CYP, Exp. 2), pink/cross-plus (CYP, Exp. 3).

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 section 3.2 above. 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 Figure 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 bio-activated organophosphate PM therefore seems to increase mortality and reduce yield.

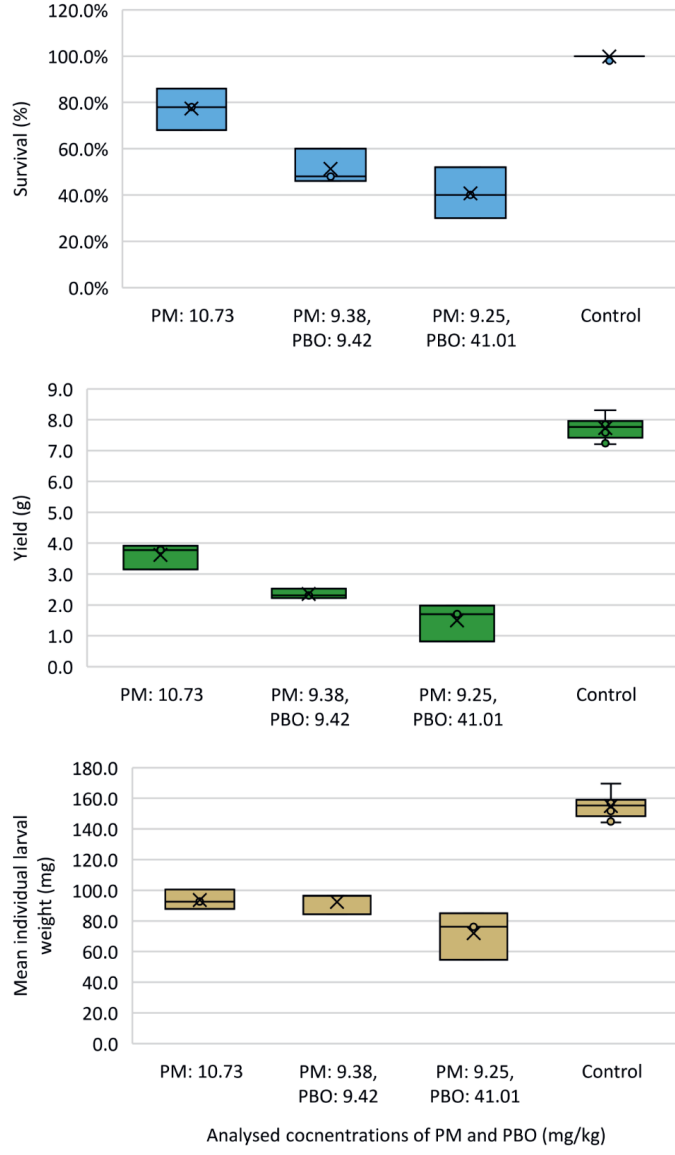

Figure 6: Survival (%), yield (g) and mean individual larval weight (mg) in response to exposure to both pirimiphos-methyl (PM) and piperonal butoxide (PBO), for the concentrations analysed (mg/kg). Box plots show median (-) and quartiles; X = arithmetic mean for n = 3 replicates per treatment, and n=9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

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 controls, even at 0.53 mg/kg (Figure 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 and Coats, 1989). The higher toxicity of deltamethrin and cypermethrin compared to permethrin is likely attributable to the presence of the α-cyano group (Khambay and Jewess, 2004). Identifying the cause for the substantial difference between toxicity of cypermethrin and deltamethrin to BSFL is less straight forward. 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 (Cetin et al., 2010, Liu and Yue, 2000, Zhang et al., 2007). This hypothesised 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 C.2. 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.

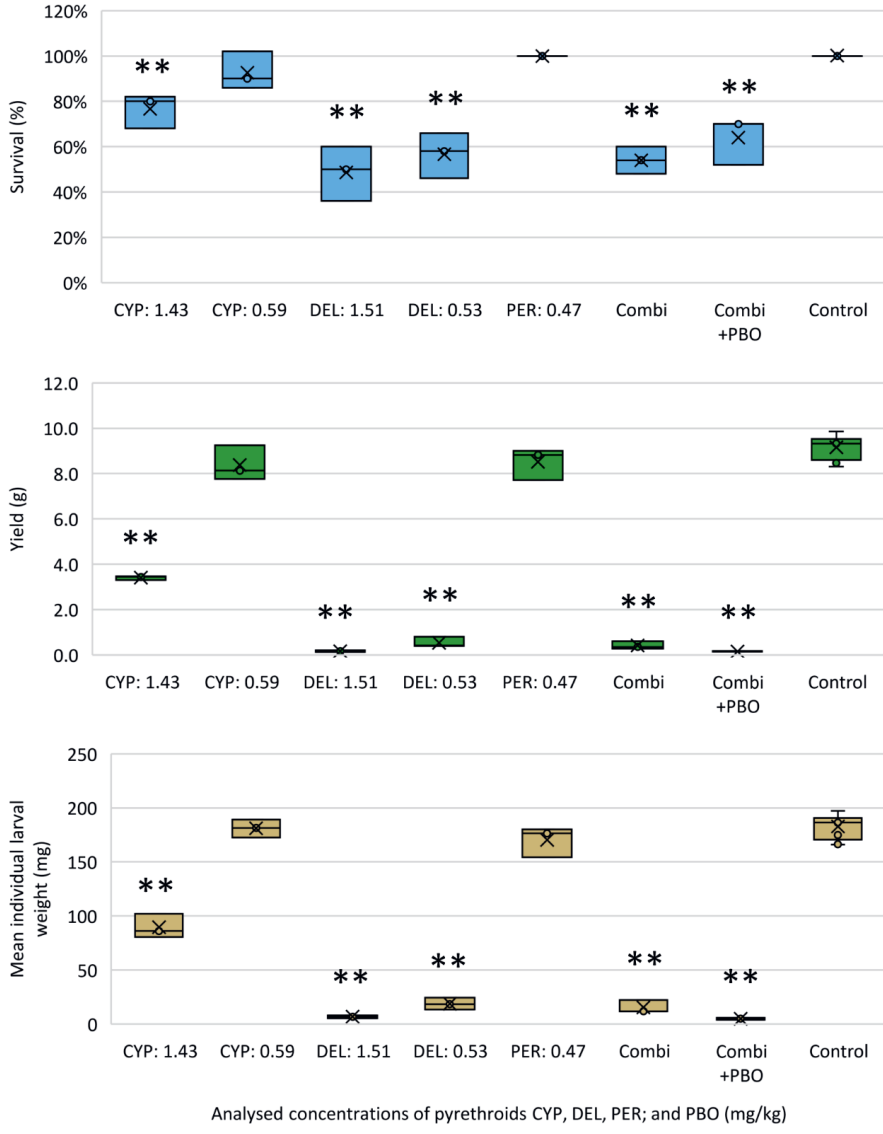

*Figure 7: Survival (%), yield (g) and mean individual larval weight (mg) in response to exposure to pyrethroids cypermethrin (CYP), deltamethrin (DEL), and permethrin (PER); all combined (combi), with or without the synergist piperonyl butoxide (PBO); for the concentrations analysed (mg/kg). Box plots 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 (Koou et al., 2014, Cakir et al., 2008, Fakoorziba et

al., 2009, Darriet and Chandre, 2013), 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 C.1, 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 ($P > 0.01$; Figure 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 does not increase mortality of BSFL when exposed to bio-activated organophosphates, as discussed in section 3.4 above.

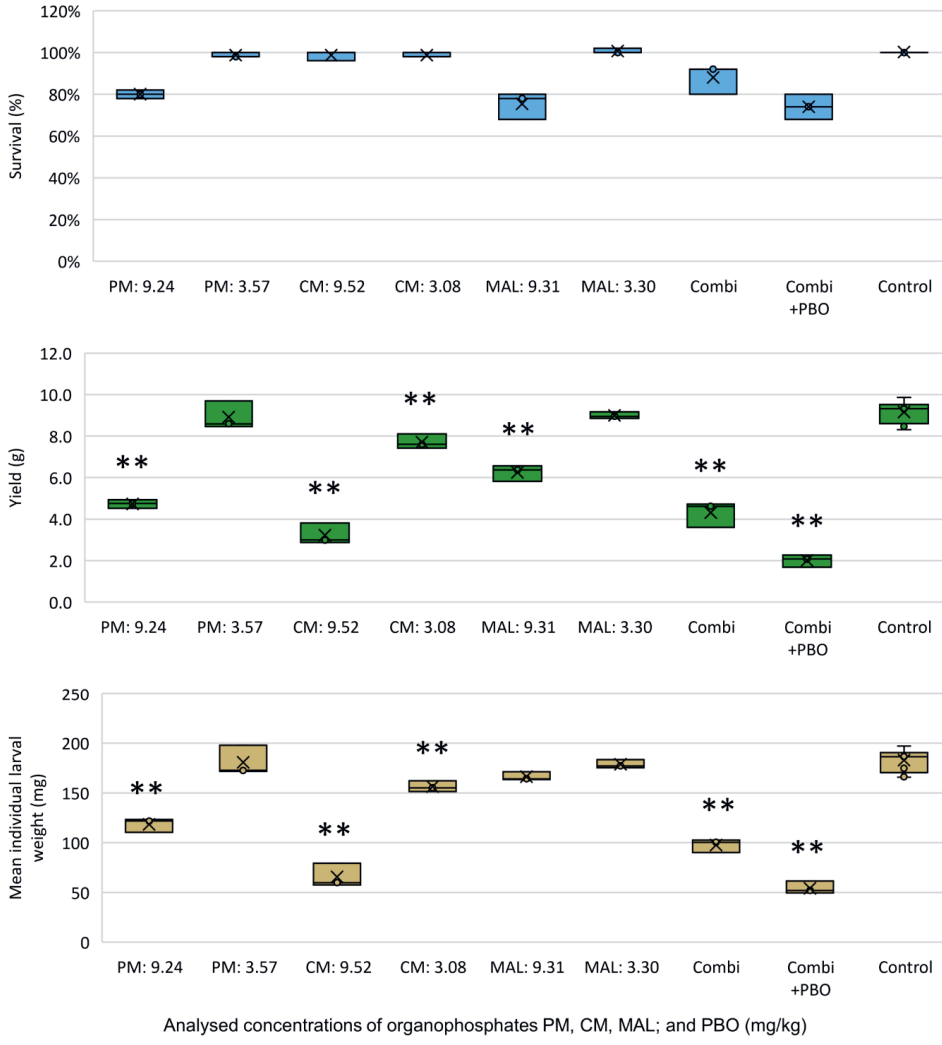

*Figure 8: Survival (%), yield (g) and mean individual larval weight (mg) in response to exposure to organophosphates pirimiphos-methyl (PM) chlorpyrifos-methyl (CM), and malathion (MAL); all combined (combi), with or without the synergist piperonyl butoxide (PBO); for the concentrations analysed (mg/kg). 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 *.*

CM and PER are no longer authorised 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 sub-lethal 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 sub-lethal 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 section 3.2 above, 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

Figure 9 shows a box-plot of individual larval weights recorded in Exp. 3. The numerical data are shown in Table B.2. 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) 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.

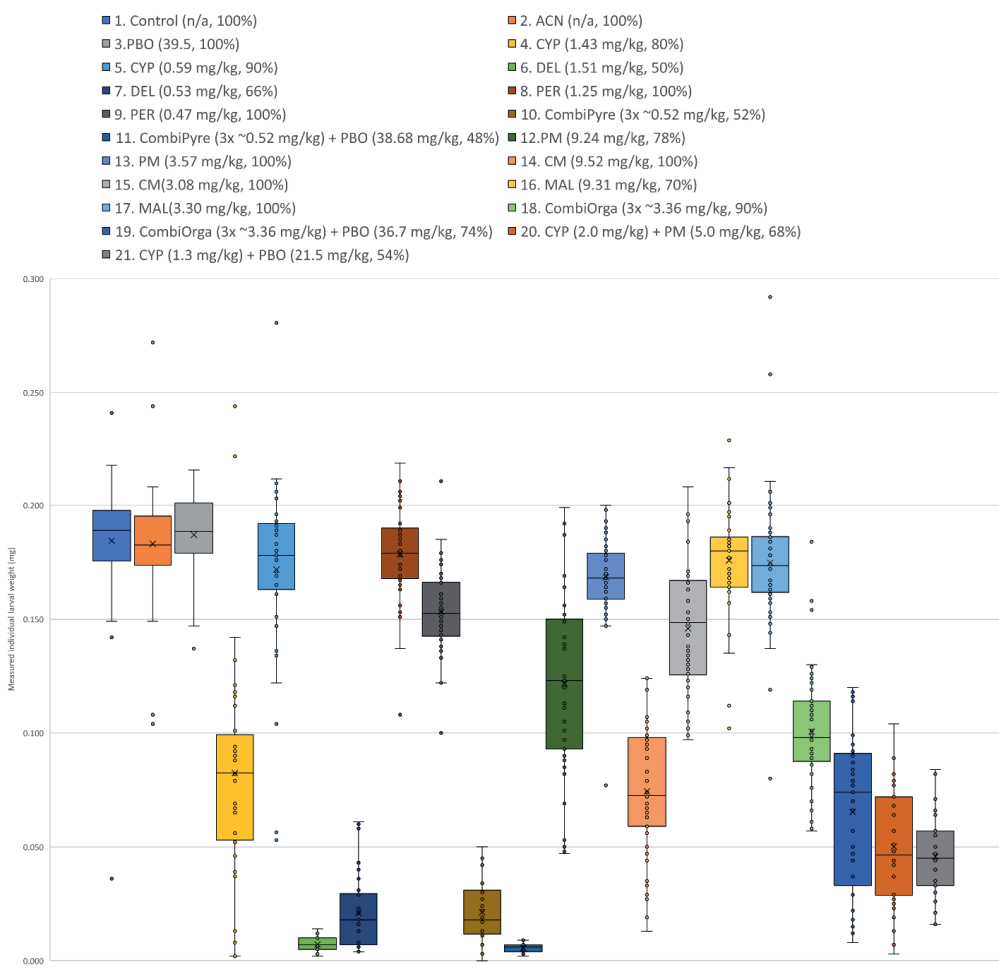

Figure 9: Results for treatments tested in Experiment 3, in terms of actual individual black soldier fly larval weight (mg) in response to exposure to tested insecticides. Pyrethroid (Pyre) substances tested were cypermethrin (CYP), deltamethrin (DEL), pirimiphos-methyl (PM); organophosphates (Orga)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

Concentrations in larvae

Table 2 presents the analysed concentrations of tested substances in the feed compared to the concentrations in the larvae. For most substances, the carry-over (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 analysed 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 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.

Table 2: Concentrations of spiked substances in BSF larval samples in Experiment 3, compared to analysed concentrations of respective substances in provided feed, and percentage of carry-over (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	**Analysed concentration feed (mg/kg)**	**Analysed 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 DEL PER	0.47 0.48 0.48	0.06 0.05 0.04	12.8% 10.4% 8.3%
CYP DEL PER PBO	0.52 0.57 0.53 38.68	0.16 0.15 0.14 7.42	30.8% 26.3% 26.4% 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	3.3	0.03	0.9%
PM CM MAL	3.68 3.01 3.03	0.02 0.01 0.00	0.5% 0.3% 0.0%
PM CM MAL PBO	3.1 3.01 2.92 36.68	0.06 0.04 0.00 0.26	1.9% 1.3% 0.0% 0.7%
CYP PM	1.95 5.25	0.19 0.10	9.7% 1.9%
CYP PBO	1.25 21.49	0.13 0.54	10.4% 2.5%

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 non-compliance 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. 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 non-compliance, 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 non-compliance 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 substate to insect biomass coupled with the low default MRLs of these substances in insects introduce a risk of non-compliance 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 sub-lethal 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 feeds consisting of a variety of ingredients from different batches or suppliers.

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

Details of the insecticide reference standards, including CAS number, insecticide class, and supplier and purity, as used for spiking and analysis are shown in Table A.1.

Table A.1: Used insecticidal substances, class and suppliers.

Substance name	CAS-No	Insecticide class	Supplier	Purity
Piperonyl butoxide	51-03-6	Synergist	LGC Standards	95.8%
Cypermethrin	52315-07-8	Pyrethroid	Sigma-Aldrich	99.0%
Permethrin	52645-53-1	Pyrethroid	Dr. Ehrenstorfer	99.7%
Deltamethrin	52918-63-5	Pyrethroid	Dr. Ehrenstorfer	99.6%
Pirimiphos-methyl	29232-93-7	Organophosphate	Dr. Ehrenstorfer	95.5%
Chlorpyrifos-methyl	5598-13-0	Organophosphate	Dr. Ehrenstorfer	99.7%
Malathion	121-75-5	Organophosphate	Dr. Ehrenstorfer	98.6%

Method and materials for chemical analyses

Extraction and sample preparation

Initial extraction

Feed samples from Exp. 1, 2 and 3 and larval samples from Exp. 3 were extracted as follows. Frozen sample material, 2.5 g (± 0.1 g), was weighed into an extraction tube, 7.5 ml of water was added, followed by 10 ml of extraction solvent (acetonitril with 1% (v/v) acetic acid) and the tubes were homogenised by vortexing for 1 min followed by 30 min of shaking end-over-end. 1.0 g of sodium acetate and 4.0 g of $MgSO_4$ were added and shaken thoroughly by hand followed by centrifugation for 5 min at 3500 rpm. The upper, organic layer was split for LC-MS/MS and GC-MS/MS as described hereafter.

Sample prep for LC-MS/MS analyses

250 µl was diluted with 250 µl of water (+0.1% acetic acid). After homogenization, the diluted extract was ready for LC-MSMS.

Sample prep for GC/MS-MS analyses

A 1.0 ml aliquot of the upper organic layer was pipetted into a dSPE-tube containing 250 mg PSA, 150 mg $MgSO_4$, 25 mg C18 and 25 mg PSA. After thorough manual shaking and centrifugation for 5 min at 13,000 rpm 200 µl of the cleaned extract was pipetted in a GC vial and 10 µL acetonitril containing 1% formic acid was added. After

homogenization the extract (equivalent with 0.25 g sample/ml) was ready for injection. In case less sample material than 2.5 g was available the extraction procedure was adjusted to correct for the lower sample weight.

Analyses

LC-MS/MS

Instrumental analysis was performed by using a Shimadzu (Kyoto, Japan) UPLC system consisting of an LC-30AD binary pump, a DGU-20A5R degasser, a SIL-30ACMP autosampler, and a CTO020AC column oven, and a Sciex (Framingham, MA, USA) 6500+ triple quad MS using multiple reaction monitoring (MRM) in the positive mode. Separation was obtained using a Waters (Etten-Leur, the Netherlands) HSS-T3, 1.8 µm, 2.1 x 100 mm UPLC column and a water/95% methanol gradient (both with 0.1% formic acid and 5 mM ammonium formate) with a cycle time of 14 minutes, and a column temperature of 45°C. Five µl of sample extract, reference solution and quality control solution was injected. The MS/MS was operated with collision gas: medium, curtain gas: 35 psi, gas 1: 55 psi, gas 2: 55 psi, an ion spray of 4500 V and a source temperature of 450°C. MSMS parameters can be found in Table A.2.

Table A.2: LC-MS/MS conditions.

Q1	**Q3**	**Substance ID**	**DP (V)**	**CE (eV)**	**CXP (V)**
198	140	^{13}C-Caffeine internal standard)	116	29	12
433.1	191	Cypermethrin	21	21	25
433.1	127		21	39	25
435	193		21	21	25
522.9	280.7	Deltamethrin	36	23	25
524.9	282.7		36	23	25
522.9	181.3		36	51	25
408.1	183.1	Permethrin	51	25	25
408.1	153.1		51	23	25
410	183		36	23	25
306.1	164.1	Pirimiphos-methyl	46	29	20
306.1	108.1		46	39	20
321.9	125.1	Chlorpyrifos-methyl	46	27	20
321.9	289.9		46	25	20
331.2	127.1	Malathion	76	17	8
331.2	99		76	35	16
331.2	285		76	10	8
356.2	177.1	Piperonyl butoxide	31	19	25
356.2	119.1		31	47	25

Legend: Q1: first quadrupole; Q3: third quadrupole; DP: declustering potential; CE: collision energy; CXP: cell exit potential.

GC-MS/MS

The insecticides were identified and quantified with an Agilent 7010B triple quad mass spectrometer equipped with an Agilent 7890B gas chromatograph and an Agilent 7693 autosampler (Agilent technologies, Santa Clara, USA). The GC was equipped with a programmed temperature vaporizer (PTV injector) which allowed injection of five µl extracts. A CL-Pesticide 30m x 0.25mm i.d. x 0.25µm film thickness analytical column (Restek, Bellefonte, USA) was used to separate the individual compounds. The MS/MS was operated in the EI+ mode at 70 eV. The source temperature was 250°C, the collision gas (N2) flow was 1.5 ml/min, the quadrupole temperature was 150°C, and the gain factor was 20 V. The monitored ions and GC-settings can be found in Table A.3. Pesticide response data were obtained and processed with MassHunter software.

Table A.3: GC-MS/MS conditions, MS/MS transitions.

Substance ID	**Rt (min)**	**CE (V)**	**Q1**	**Q3**
PCB-198 (internal standard)	17.70	14	430	360
Cypermethrin	20.16	28	181	152
	20.16	2	165	127
	20.16	2	163	127
	20.32	28	181	152
	20.45	28	181	152
Deltamethrin	21.08	2	253	174
	21.08	20	253	93
	21.25	2	253	174
	21.25	20	253	93
Permethrin	18.60	16	183	168
	18.60	10	163	127
	18.83	16	183	168
	18.83	10	163	127
Pirimiphos-methyl	12.93	6	305	180
	12.93	16	290	151
Chlorpyrifos-methyl	12.49	16	286	271
	12.49	26	286	93
Malathion	13.77	2	173	127
	13.77	16	173	99
Piperonyl butoxide	16.32	16	176	131
	16.32	20	176	117

Legend: Rt: retention time; CE: collision energy; Q1: precursor ion; Q3: product ion

The PTV-injector of the gas chromatograph was used in the solvent vent mode. Five µl of extract was injected at a velocity of 69 µl/sec at 70°C (0.065 min) with 750°C/min to 275°C (1.5 min.), with 750°C/min. to 350°C (2 min). The oven temperature program runs from 60°C (2 min) with 20°C/min to 150°C, with 10°C/min to 280°C, with 25°C/min to 320°C (2 min) and then with 100°C/min to 60°C and the carrier gas was helium with 1 ml/min.

Quality control (QC) results feed

Results of the quality control for verifying homogeneity of the spiked substances in the feed in Exp. 1 are shown in Table A.4. All four treatments were sufficiently homogenous

(RSD ≤10%) and analysed concentrations did not substantially deviate from intended concentrations (<10%).

Table A.4: 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. Mean concentrations of n=10 samples in mg/kg. Relative standard deviation (RSD) calculated as the standard deviation of analysed samples per treatment, divided by the mean. Concentrations of CYP corrected for the mean recovery of both treatments containing CYP.

Parameter	**Spiked substance(s)**			
	CYP	**CYP + PBO**	**PM**	**PM + PBO**
Intended concentration (mg/kg)	8.0	0.5 + 0.5	2.50	10.0 + 10.0
Recovery (%)	81	84 + 92	94	95 + 90
RSD (%)	4.0	4.6 + 3.5	5.5	2.6 + 2.3
Mean analysed concentration (dry) (mg/kg)	20.0	1.16 + 1.46	7.16	26.8 + 26.9
Calculated concentration (wet) (mg/kg) *	(6.98)	(0.40) + 0.51	2.50	9.38 + 9.42
Corrected concentration (wet) (mg/kg)	8.49	0.49	-	-

**Between brackets: concentration before correction.*

QC results for concentrations of substances in the diet of the three experiments are shown in Table A.5. For non-polar substances, such as pyrethroids, high fat concentrations in the analysed matrix may lower the recovery during extraction for analytical purposes. Therefore, CYP concentrations in the feed were corrected for recovery, using the mean percentage of both QC samples containing this substance.

Table A.5: Recovery percentages of analytical series to verify concentration of spiked substances in black soldier fly larvae diet. Treatments in experiments 1 and 2 were analysed in analytical series 1. Analytical series 2 concerned the repetition of 4 treatments from both experiments to check for substantial deviations observed in series 1. Treatments in experiment 3 were analysed in series 3.

Spiked substance	**Recovery (%)**		
	Low	**High**	**Mean**
Analytical series 1			
Cypermethrin	89	82	85
Pirimiphos-methyl	100	91	96
Piperonyl butoxide	93	88	91
Analytical series 2			
Cypermethrin	95	-	-
Pirimiphos-methyl	106	-	-
Analytical series 3			
Cypermethrin	-	101	-
Deltamethrin	-	102	-
Permethrin	-	102	-
Pirimiphos-methyl	-	78	-
Chlorpyrifos-methyl	-	91	-
Malathion	-	89	-
Piperonyl butoxide	-	93	-

Quality control (QC) results larvae

QC results for larvae are shown in Table A.6. All recovery percentages as presented in this Table were acceptable, and concentrations were therefore not corrected.

Table A.6: Recovery percentages of analytical series to verify concentration of spiked substances in blank black soldier fly larvae.

Spiked substance	**Recovery (%)**			
	Low	**Intermediate**	**High**	**Mean**
CYP	-	81, 87	105	91
DEL	-	70	-	-
PER	86	88		87
PM	73	92		82
CM	82	88		85
MAL	86	87		86
PBO	85, 87	84		85

Appendix B: Overview of experimental results

*Table B.1: Overview of results from three executed experiments. Intended and analysed concentration in mg/kg for each treatment, and ratio between these two as a %. Experimental results in terms of survival (%), total larval biomass (yield, g), and mean individual larval weight (mg). The latter is calculated as yield divided by number of surviving larvae * 1000. Results from n=3 replicates per treatment.*

Treatment (intended concentration (mg/kg))	**Analysed concentration (mg/kg)**	**Final / intended concentration (%)**	**Survival (%)**	**Yield (g)**	**Mean ind. Larv. weight (mg)**
Experiment 1					
Control (n/a)			100.0% 100.0% 100.0%	7.59 7.84 7.21	151.8 156.8 144.2
Control – acetonitrile [ACN] (n/a)			100.0% 100.0% 100.0%	8.01 7.9 7.68	160.2 158.0 153.6
Piperonyl butoxide (PBO) (40.00)	36.20	90%	98.0% 100.0% 100.0%	8.31 7.77 7.24	169.6 155.4 144.8
Cypermethrin (CYP) (0.50)	0.55	110%	100.0% 96.0% 100.0%	8.31 7.77 7.24 *	133.6 150.2 135.2
CYP (2.00)	1.83	92%	78.0% 72.0% 82.0% *	1.63 2.48 2.35 *	41.8 68.9 57.3 *
CYP (8.00)	8.49	106%	50.0% 58.0% 50.0% *	0.25 0.26 0.28 *	10.0 9.0 11.2 *
CYP (0.50) / PBO (0.50)	0.49 0.51	98% 102%	100.0% 100.0% 100.0%	5.65 6.74 6.99 *	113.0 134.8 139.8 *
CYP (0.50) / PBO (5.0)	0.44 4.37	88% 87%	96.0% 100.0% 96.0%	5.15 5.67 5.83 *	107.3 113.4 121.5 *
CYP (0.50) / PBO (10.0)	0.50 9.93	100% 99%	100.0% 96.0% 96.0%	4.93 4.72 5.10 *	98.6 98.3 106.3 *
CYP (2.00) / PBO (2.0)	1.80 1.78	90% 89%	78.0% 84.0% 82.0% *	1.38 1.89 1.53 *	35.4 45.0 37.3 *
CYP (2.00) / PBO (20.0)	2.10 20.44	105% 102%	82.0% 72.0% 86.0% *	0.76 0.70 1.05 *	18.5 19.4 24.4 *
CYP (2.00 / PBO (40.0)	1.94 41.93	97% 105%	70.0% 76.0% 80.0% *	0.56 0.54 0.53 *	16.0 14.2 13.3 *

Table B.1, continued.

Treatment (intended concentration (mg/kg))	Analysed concentration (mg/kg)	Final / intended concentration (%)	Survival (%)	Yield (g)	Mean ind. Larv. weight (mg)
Experiment 1					
Pirimiphos-methyl (PM) (2.50)	2.50	100%	100.0% 100.0% 100.0%	7.77 7.82 7.10	155.4 156.4 142.0
PM (5.00)	4.84	97%	100.0% 100.0% 100.0%	7.17 7.03 6.77 *	143.4 140.6 135.4 *
PM (10.00)	10.73	107%	68.0% 78.0% 86.0% *	3.15 3.92 3.78 *	92.6 100.5 87.9 *
PM (10.00) / PBO (10.0)	9.38 9.42	94% 94%	48.0% 46.0% 60.0% *	2.31 2.22 2.53 *	96.3 96.5 84.3 *
PM (10.00) / PBO (40.00)	9.25 41.01	92% 103%	52.0% 40.0% 30.0% *	1.98 1.70 0.82 *	76.2 85.0 54.7 *
Experiment 2					
Control (n/a)			100.0% 100.0% 100.0%	7.56 7.34 8.19	151.1 146.8 163.8
Control – can (n/a)			98.0% 100.0% 100.0%	6.95 8.35 7.07	141.8 167.0 141.3
PBO (40.0)	38.98	97%	98.0% 100.0% 100.0%	8.54 7.43 8.12	174.3 148.6 162.4
CYP (1.00)	1.07	107%	96.0% 94.0% 92.0% *	6.96 6.93 6.09	144.9 147.4 132.3
CYP (4.50)	4.01	89%	78.0% 66.0% 62.0% *	1.33 1.36 1.12 *	34.0 41.3 36.0 *
CYP (0.20) / PBO (4.00)	0.18 3.68	89% 92%	102.0% 100.0% 98.0%	7.99 7.63 8.22	156.6 152.5 167.7
CYP (1.00) / PBO (20.0)	0.92 18.03	92% 90%	96.0% 86.0% 92.0% *	5.03 4.06 4.84 *	104.8 94.3 105.1 *
PM (7.50)	7.33	98%	84.0% 88.0% 78.0% *	5.09 4.94 4.46 *	121.2 112.3 114.4 *
CYP (0.20) / PM (0.50)	0.16 0.45	81% 90%	100.0% 100.0% 100.0%	8.23 8.21 7.78	164.7 164.2 155.6

Table B.1, continued.

Treatment (intended concentration (mg/kg))	Analysed concentration (mg/kg)	Final / intended concentration (%)	Survival (%)	Yield (g)	Mean ind. Larv. weight (mg)
Experiment 2					
CYP (0.20) / PM (0.50) / PBO (4.00)	0.18 0.50 3.78	88% 100% 94%	98.0% 100.0% 100.0%	8.80 8.29 8.55	179.7 165.7 171.0
CYP (2.00) / PM (5.00) / PBO (40.00)	1.92 4.89 39.24	96% 98% 98%	84.0% 80.0% 74.0% *	1.02 1.50 1.10 *	24.3 37.6 29.6 *
Experiment 3					
Control (n/a)			100.0% 100.0% 100.0%	9.32 8.74 9.39	186.40 174.80 187.80
Control – ACN (n/a)			100.0% 100.0% 100.0%	9.32 8.31 9.60	186.40 166.20 192.00
PBO (40.3)	39.52	98%	100.0% 102.0% 100.0%	9.45 8.46 9.86	189.00 165.88 197.20
CYP (1.57)	1.43	91%	80.0% 68.0% 82.0% *	3.44 3.47 3.30 *	86.00 102.06 80.49 *
CYP (0.52)	0.59	113%	90.0% 86.0% 102.0%	7.76 8.13 9.25	172.44 189.07 181.37
Deltamethrin (DEL) (1.57)	1.51	96%	50.0% 36.0% 60.0% *	0.20 0.12 0.16 *	8.00 6.67 5.33 *
DEL (0.52)	0.53	102%	66.0% 58.0% 46.0% *	0.80 0.39 0.42 *	24.24 13.45 18.26 *
Permethrin (PER) (1.57)	1.25	79%	**	**	**
PER (0.52)	0.47	90%	100.0% 100.0% 100.0%	7.71 9.01 8.82	154.20 180.20 176.40
CYP (0.52) / DEL (0.52) / PER (0.52)	0.47 0.48 0.48	91% 92% 92%	54.0% 60.0% 48.0% *	0.60 0.35 0.28 *	22.22 11.67 11.67 *
CYP (0.52) / DEL (0.52) / PER (0.52) / PBO (31.4)	0.52 0.57 0.53 38.68	100% 110% 101% 123%	52.0% 70.0% 70.0% *	0.15 0.17 0.13 *	5.77 4.86 3.71 *
PM (10.08)	9.24	92%	78.0% 80.0% 82.0% *	4.75 4.93 4.52 *	121.79 123.25 110.24 *

Table B.1, continued.

Treatment (intended concentration (mg/kg))	Analysed concentration (mg/kg)	Final / intended concentration (%)	Survival (%)	Yield (g)	Mean ind. Larv. weight (mg)
Experiment 3					
PM (3.36)	3.57	106%	100.0% 98.0% 98.0%	8.59 8.46 9.70	171.80 172.65 197.96
Chlorpyrifos-methyl (CM) (10.08)	9.52	94%	96.0% 100.0% 100.0%	3.81 2.99 2.88 *	79.38 59.80 57.60 *
CM (3.36)	3.08	92%	98.0% 100.0% 98.0%	7.60 8.11 7.42 *	155.10 162.20 151.43 *
Malathion (MAL) (10.08)	9.31	92%	68.0% 80.0% 78.0% *	5.82 6.57 6.37 *	171.18 164.25 163.33
MAL (3.36)	3.30	98%	100.0% 100.0% 102.0%	8.86 9.17 8.95	177.20 183.40 175.49
PM (3.36) / CM (3.36) / MAL (3.36)	3.68 3.01 3.03	109% 90% 90%	92.0% 92.0% 80.0% *	4.72 4.62 3.60 *	102.61 100.43 90.00 *
PM (3.36) / CM (3.36) / MAL (3.36) / PBO (42.92)	3.10 3.01 2.92 36.68	92% 90% 87% 91%	74.0% 68.0% 80.0% *	2.27 1.68 2.08 *	61.35 49.41 52.00 *
CYP (2.00) / PM (5.00)	1.95 5.25	97% 105%	68.0% 58.0% 80.0% *	1.47 1.57 2.30 *	43.24 54.14 57.50 *
CYP (1.30) / PBO (26.0)	1.25 21.49	96% 83%	66.0% 82.0% 78.0% *	1.75 1.51 1.66 *	53.03 36.83 42.56 *

**: Significantly different compared to pooled controls, Mann-Whitney U post-hoc test (P ≤ 0.01)*
***: Not presented here due to quality control issues.*

Table B.2: Overview of individual larval weight (mg), based on individually weighed larvae in Exp. 3. Mean and standard deviation for the number of surviving larvae in the first replicate of each treatment (out of n=50 at start of experiment).

Treatment (analysed concentration (mg/kg))	**Number of surviving larvae in first replicate**	**Individual larval weight**
Control (n/a)	50	184 ± 28
Control – ACN (n/a)	50	183 ± 26
Piperonyl butoxide (PBO) (39.52)	50	187 ± 18
Cypermethrin (CYP) (1.43)	40	82 ± 49
CYP (0.59)	45	172 ± 38
Deltamethrin (DEL) (1.51)	25	7 ± 3
DEL (0.53)	33	21 ± 17
Permethrin (PER) (1.25)	*	*
PER (0.47)	50	153 ± 19
CYP (0.47) / DEL (0.48) / PER (0.48)	27	21 ± 13
CYP (0.52) / DEL (0.57) / PER (0.53) / PBO (31.68)	26	6 ± 2
Pirimihphos-methyl (PM) (9.24)	39	122 ± 41
PM (3.57)	50	169 ± 19
Chlorpyrifos-methyl (CM) (9.52)	50	74 ± 28
CM (3.08)	50	146 ± 27
Malathion (MAL) (9.31)	35	176 ± 26
MAL (3.30)	50	175 ± 31
PM (3.68) / CM (3.01) / MAL (3.03)	46	101 ± 26
PM (3.10) / CM (3.01) / MAL (2.92) / PBO (36.68)	37	65 ± 35
CYP (1.95) / PM (5.25)	34	50 ± 26
CYP (1.25) / PBO (21.49)	33	46 ± 18

**: Not presented here due to quality control issues.*

Appendix C: Monitoring data for insect feeds

Table C.1: Monitoring data from project partner ForFarmers for commercial feed ingredients, including product type, country of origin, and sample date. The materials were sampled during the 2020 and 2021 harvests. Actual sampled products were derivative (processed) of product types shown, MRLs therefore do not directly apply without processing factors. Concentrations in mg/kg of cypermethrin (CYP), deltamethrin (DEL), pirimiphos-methyl (PM), chlorpyrifos-methyl (CM), and piperonyl butoxide (PBO). All samples were negative for permethrin and malathion. The highest quantified concentration of each substance is highlighted grey.

#	Product type	Country of origin	Sample date	CYP	DEL	PM	CM	PBO
1	Wheat	FRA	2020-12-22	0.011	-	-	-	0.014
2	Barley	NLD	2021-01-04	0.25	0.17	0.27	-	0.35
3	Wheat		2020-11-16	-	0.18	-	-	-
4	Wheat	FRA	2021-01-12	-	0.017	0.8	-	0.099
5	Maize/corn	NLD	2021-01-11	-	-	-	-	0.031
6	Cocoa beans	NLD	2021-01-19	0.015	-	-	-	-
7	Barley	BEL	2021-03-11	0.15	-	-	0.027	0.5
8	Wheat	BEL	2021-04-01	0.25	0.036	0.25	-	1.2
9	Barley		2021-03-30	-	-	0.017	-	-
10	Wheat	FRA	2021-04-14	0.1	0.066	0.45	-	1.5
11	Wheat	NLD	2021-04-30	0.56	0.065	0.038		3.1
12	Cocoa beans	EU	2021-05-14	0.037	-	-	0.022	-
13	Wheat	EU	2021-05-19	0.13	0.09	0.02	-	0.67
14	Maize/corn	NLD	2021-05-27	-	0.059	0.017	-	0.56
15	Maize/corn	EU	2021-06-03	-	-	0.038	-	0.11
16	Barley	NLD	2021-06-04	0.27	0.087	0.026	-	0.9
17	Sunflower seeds	NLD	2021-06-23	-	-	-	-	0.022
18	Oat	EU	2021-07-07	-	-	0.039	-	-
19	Wheat	DEU	2021-05-25	-	-	0.56	0.02	-
20	Wheat	NLD	2021-07-27	0.027	-	0.1	-	0.15
21	Soy	BRA	2021-07-27	-	-	-	-	0.091
22	Maize/corn		2021-08-17	-	1.1	-	-	4.2
23	Maize/corn	BRA	2021-09-02	-	-	0.04	-	0.019
24	Maize/corn	NLD	2021-09-07	-	-	0.048	-	-
25	Beans		2021-09-14	-	-	-	-	0.015
26	Maize/corn	POL	2021-09-13	-	-	0.019	-	-
27	Maize/corn	EU	2021-10-05	-	0.019	0.036	-	-
28	Wheat	DEU	2021-09-29	-	-	0.081	-	0.048
29	Maize/corn	EU	2021-11-02	-	0.014	-	-	0.12
30	Wheat	BEL	2021-11-10	0.46	0.037	-	-	1.1
31	Wheat		2021-10-13	-	-	-	-	0.04

–: indicates that the concentration was below the limit of quantification (<LOQ).

Table C.2: 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. The materials were sampled during 2017-2022. Actual sampled products were derivative (processed) of product types shown, MRLs therefore do not directly apply without processing factors. Concentrations in mg/kg of cypermethrin (CYP), deltamethrin (DEL), pirimiphos-methyl (PM), chlorpyrifos-methyl (CM), and piperonyl butoxide (PBO). The highest quantified concentration of each substance is highlighted blue.

#	Product type	Sample date	CYP	DELT	PM	CM	PBO
1	Compound feed (wheat/potato)	2022-02-14	0.080	-	0.020	-	0.450
2	Compound feed (wheat/potato)	2022-01-13	-	-	-	-	0.100
3	Corn pellets	2021-12-30	-	-	0.010	-	
4	Compound feed (wheat/potato)	2021-12-22	-	-	-	-	0.022
5	Compound feed (wheat/potato)	2021-11-24	-	-	-	-	0.033
6	Corn pellets	2021-10-13	-	-	-	-	0.014
7	Compound feed (wheat/potato)	2021-08-30	-	-	-	-	0.010
8	Bran (fine)	2021-07-29	-	-	-	-	0.011
9	Compound feed (wheat/potato)	2021-07-02	-	-	-	-	0.039
10	Compound feed (wheat/potato)	2021-06-14	-	-	-	-	0.020
11	Corn pellets	2021-05-31	-	0.180	0.019	-	0.130
12	Compound feed (wheat/potato)	2021-05-27	-	-	-	-	0.014
13	Compound feed (wheat/potato)	2021-04-06	-	-	-	-	0.013
14	Compound feed (wheat/potato)	2021-03-18	-	-	-	-	0.011
15	Compound feed (wheat/potato)	2021-01-26	-	-	-	-	0.021
16	Compound feed (wheat/potato)	2021-01-26	0.015	-	-	-	0.010
17	Compound feed (wheat/potato)	2020-07-30	-	-	-	-	0.012
18	Corn pellets	2020-07-17	-	0.022	-	-	0.250
19	Compound feed (wheat/potato)	2020-07-17	0.023	-	-	-	0.470
20	Compound feed (wheat/potato)	2020-04-20	0.043	-	0.029	-	0.290
21	Wheat yeast concentrate	2020-04-17	0.350	-	0.099	-	0.490
22	Wheat yeast concentrate	2020-03-11	0.042	0.026	-	-	0.600
23	Wheat yeast concentrate	2020-02-17	0.026	0.019	-	-	0.410
24	Beet pulp powder	2020-02-04	-	-	-	-	0.011
25	Wheat yeast concentrate	2020-01-30	0.035	-	0.020	-	0.350
26	Wheat yeast concentrate	2019-12-23	0.058	-	-	-	0.260
27	Wheat yeast concentrate	2019-12-03	0.049	-	0.030	-	0.190
28	Wheat yeast concentrate	2019-11-07	0.012	-	0.010	-	0.094
29	Compound feed (wheat/potato)	2019-11-04	0.024	-	0.120	-	0.180
30	Wheat yeast concentrate	2019-10-21	0.030	-	-	-	0.120
31	Compound feed (wheat/potato)	2019-10-11	0.034	0.016	-	-	0.190
32	Wheat yeast concentrate	2019-06-17	0.045	0.017	0.030	-	0.026
33	Beet pulp powder	2019-06-12	-	-	-	-	0.044
34	Compound feed (wheat/potato)	2019-05-27	0.017	-	0.034	-	0.065
35	Wheat yeast concentrate	2019-05-21	0.077	0.016	0.012	-	0.180
36	Wheat yeast concentrate	2019-04-24	0.023	-	0.017	-	0.110
37	Beet pulp powder	2019-04-24	-	-	0.017	-	0.016
38	Compound feed (wheat/potato)	2019-04-11	-	-	-	-	0.017
39	Wheat yeast concentrate	2019-03-21	0.012	-	0.013	-	0.057

Table C.2, continued.

#	Product type	Sample date	CYP	DELT	PM	CM	PBO
40	Beet pulp powder	2019-03-04	0.010	-	0.033	-	0.069
41	Beet pulp powder	2019-02-15	-	-	-	-	0.013
42	Wheat yeast concentrate	2019-02-08	0.070	0.014	-	-	0.350
43	Bran (coarse)	2019-02-08	-	0.086	1.100	-	1.500
44	Beet pulp powder	2019-01-24	-	-	-	-	0.023
45	Bran (fine)	2019-01-21	-	-	-	-	0.011
46	Wheat yeast concentrate	2019-01-02	0.044	-	0.025	-	0.340
47	Compound feed (wheat/potato)	2019-01-02	-	-	-	-	0.025
48	Wheat yeast concentrate	2018-12-05	0.018	-	0.031	-	0.190
49	Compound feed (confectionary)	2018-11-02	-	0.860	-	0.110	4.000
50	Beet pulp powder	2018-10-26	-	-	-	-	0.016
51	Wheat yeast concentrate	2018-10-23	0.020	-	-	-	0.210
52	Wheat yeast concentrate	2018-09-21	-	-	0.016	-	0.250
53	Bran (coarse)	2018-08-05	-	-	-	-	0.036
54	Wheat yeast concentrate	2018-08-03	0.089	-	-	-	0.370
55	Compound feed (wheat/potato)	2018-07-17	-	-	-	-	0.016
56	Wheat yeast concentrate	2018-06-13	0.079	-	-	-	0.290
57	Bran (coarse)	2018-06-06	-	-	-	-	0.016
58	Compound feed (confectionary)	2018-04-16	0.019	-	0.320	0.024	0.068
59	Compound feed (confectionary)	2018-04-05	0.016	-	0.320	-	0.049
60	Compound feed (confectionary)	2018-03-02	-	-	-	0.300	0.036
61	Compound feed (confectionary)	2018-02-27	-	-	-	0.010	0.068
62	Wheat yeast concentrate	2018-02-20	0.041	-	-	-	0.170
63	Compound feed (confectionary)	2018-01-02	-	-	0.081	0.044	0.025
64	Wheat yeast concentrate	2017-12-28	0.020	-	-	-	0.110
65	Compound feed (confectionary)	2017-11-24	-	-	-	0.078	0.025
66	Compound feed (confectionary)	2017-11-16	-	-	-	0.021	0.066
67	Wheat yeast concentrate	2017-11-06	0.017	-	-	-	0.110
68	Compound feed (confectionary)	2017-11-03	0.025	-	-	0.049	0.026
69	Compound feed (confectionary)	2017-09-12	0.010	0.010	-	-	0.120
70	Compound feed (confectionary)	2017-08-30	-	-	0.018	-	0.270
71	Wheat yeast concentrate	2017-07-24	0.044	-	-	-	0.210

–: indicates that the concentration was below the limit of quantification (<LOQ).

Appendix D: Small-scale neonate larvae experiment

Methodology

For the experiment with the neonate larvae aged 1 to 7 days, a substantially larger amount of wet feed was required for each replicate. Due to health and safety concerns, spiking this amount by creating a slurry with methanol was not feasible. Therefore, the slurry was made directly with water one day prior to the start of the experiment, to minimize deterioration of the feed. The experiment consisted of six treatments: a blank control and one containing only PBO (PBO, 20.0 mg/kg); cypermethrin (CYP, 0.5 mg/kg); pirimiphs-methyl (PM, 5.0 mg/kg), and; two treatments each combining CYP or PM at the same concentrations but with PBO at 10.0 and 20.0 mg/kg, respectively. The treatments were performed in duplicate. For each treatment, a batch of 2.5 kg of wet feed was prepared by creating a slurry of 875 g of dry feed (the same feed as used for the P2 larvae) and 1625 ml tap water. The required volume of the laboratory standard insecticides (same as P2 larvae, see Table 2) were added, after which the slurries were mixed using a UM 12 table-top mixer (Stephan Machinery GmbH, Hameln, Germany) at 1500 rpm for 2 min. The wet feed was directly deposited into the plastic, rectangular replicate containers (16,5 x 16,5 x 12,5 cm) and left in cooled storage (4-7°C) overnight before being transported to the facility where the experiment would take place (Bestico N.V., Berkel en Rodenrijs, The Netherlands).

For this experiment, 500 mg (maximum allowed deviation of 10 mg) of neonate larvae which had hatched on that day were added to each replicate container. The experimental procedures were the same as for the P2 larvae, but no moisture was added after the start of the experiment. On experimental day 8, the larvae were removed from the replicate containers using metals spoons and tweezers. The total recovered larval biomass was weighed, and for three sub-samples of ~1.0 g per replicate the number (n) of larvae was counted. The mean of these sub-samples was assumed to be representative for the replicate and was extrapolated to the total biomass to calculate the total number of larvae post-experiment. This figure was compared against the number of larvae on experimental day 1 (assuming n=58,000 larvae per 1.0 g of neonate larvae, based on internal figures of the rearing facility) and expressed as a survival percentage. Due to the small scale (n=2 replicates), the parameter mean of each treatment is presented directly, and statistical analysis is omitted.

Results and discussion

Results of this experiment are shown in Table D.1. These results show that these younger larvae (D1-7) are much more susceptible to both substances than the older larvae (D7-14). Survival and yield were impacted by CYP, but even more so for PM – more severe than would be expected based on the results with the older larvae. We assume that this overall higher susceptibility also extends to other insecticidal substances, but more research is needed to statistically verify these results and to test other substances.

Table D.1: Results of small-scale experiment with insecticides using neonate larvae (D1-7). Mean for total larval weight after washing and drying (yield in g) and survival of larvae as a percentage of estimated number of larvae on experimental day 1 (survival in %).

Number	**Treatment (substances)**	**Spiked concentrations (mg/kg)**	**Yield (g)**	**Survival (%)**
1	Control (blank)	n/a	168.8	98.2
2	Control (piperonyl butoxide (PBO))	20.0	183.2	101.7
3	Cypermethrin (CYP)	0.5	180.6	60.7
4	CYP + PBO	0.5 + 10.0	119.2	50.4
5	Pirimiphos-methyl (PM)	5.0	53.9	11.7
6	PM + PBO	5.0 + 20.0	29.3	10.3

Chapter 5

Lethal and sublethal effects of chronic exposure to insecticide residues on reared *Alphitobius diaperinus*

(Alphitobius diaperinus adult beetle. Photo by author.)

Abstract

Edible insects such as lesser mealworm (*Alphitobius diaperinus*) are a promising new protein source for food and feed. The feed substrate on which these insects are reared may be contaminated with residues of insecticides originating from agricultural products that may impact insect performance. In this study, two generations of *A. diaperinus* were chronically exposed to spinosad (2.0 and 0.2 mg/kg) and imidacloprid (0.1 and 0.01 mg/kg) in the substrate. The aim was to determine sub-lethal effects on performance measures (total biomass (yield), mean individual weight, number of alive individuals) of larvae, pupae, and adult beetles, as well as pupation and eclosion. Exposure to spinosad at 2.0 mg/kg resulted in significant adverse effects on most performance measures of larvae, of both generations. Imidacloprid caused a reduction in yield and mean individual weight of the larvae as compared to the control at 0.1 mg/kg, while an increase in those measures was observed at 0.01 mg/kg. Significant adverse effects on adult beetles were only observed for imidacloprid at 0.1 mg/kg, and no significant effects of this insecticide on pupation and eclosion were observed. The concentrations of tested substances in larval samples were negligible for both generations, however, transfer from substrate to larval biomass was higher in the offspring generation relative to the parent generation. More research is needed to fully assess the hazard of insecticide residues to cause sub-lethal effects on *A. diaperinus*, for which method development for more cost-efficient designs is required.

Introduction

Reared insects are increasingly seen as a suitable alternative protein source for food and feed (Sogari et al. 2019, Van Huis 2020, Hawkey et al. 2021, Van Huis 2021). One species that has received attention as a promising food source is the lesser mealworm (LMW, *Alphitobius diaperinus* (Panzer); Coleoptera: Tenebrionidae). The LMW is one of the first insects that have been authorized as a novel food (Regulation (EU) 2023/58), following evaluation by the European Food Safety Authority (EFSA) (EFSA 2022). A recent study showed that LMW in its larval stage was susceptible to insecticide residues that may be present in the diet on which these insects are reared (Meijer et al. 2022a). These findings underline the need for more research on the effects of insecticide residues on reared LMW. One aspect that is of particular interest, is the potential effect of insecticide residues on LMW in case of exposure to a chronic sublethal concentration. Sublethal effects are effects on individuals within a population that survive exposure: these effects can manifest in a wide range of physiological or behavioural aspects, including development, adult longevity, fecundity, sex ratio, and mobility (Desneux et al. 2007). For the reared insect industry specifically, reduced long-term yields as a result of sublethal effects would be of economic concern, and potential accumulation of insecticidal substances in the insect biomass may pose a food safety risk.

In the study of Meijer et al. (2022a), larval LMW were reared for 14 days on substrates that had been spiked with selected insecticides. Spiked concentrations of these substances in the substrate were equal to the respective maximum residue limit (MRL) of the selected insecticides in feed. The post-trial concentration of these insecticides in the larvae were equal to or below the limit of quantification (LOQ), suggesting that bioaccumulation in the larvae did not occur, and there are no food safety concerns regarding LMW exposed to these substances at spiked concentrations. However, a significant reduction in total yield was caused by imidacloprid at 0.1 mg/kg (68.4 ± 4.0 % of mean control value) and spinosad at 2.0 mg/kg (83.7 ± 4.2 % of mean control value). A study on LMW as a pest in poultry houses also found imidacloprid and spinosad to be effective in controlling adult and larval LMW; it was highlighted that these substances might be especially suitable against populations that have developed resistance against pyrethroid and organophosphate insecticides (Singh and Johnson 2015). The efficacy of spinosad to control several coleopteran grain pests (Nayak et al. 2005, Huang and Subramanyam 2007, Hertlein et al. 2011), and *A. diaperinus* in particular (Lambkin and Rice 2007, Mustač et al. 2013, Lambkin and Furlong 2014, Tomberlin et al. 2014, Singh and Johnson 2015, Zafeiriadis et al. 2021), are well documented. Spinosad has been found to cause sublethal effects in a large variety of insects, but research on effects other than on mortality appear to have primarily focused on species in orders other than Coleoptera (Biondi et al. 2012).

To date, the active substance imidacloprid is no longer approved as an insecticide in the European Union (EU; Regulation (EU) No 485/2013), in part due to this neonicotinoid's sublethal effects on honeybees and other pollinators (EFSA 2013a, b, Fryday et al.

2015). Under certain conditions, seeds coated with imidacloprid could still be used in permanent greenhouses (Regulation (EU) 2018/783). The EU-wide approval for this insecticide expired in December 2020 (Regulation (EU) 2020/1643), but several EU countries (e.g., Belgium) had temporarily re-authorized some neonicotinoids for certain uses within their countries, using emergency derogation powers of Article 53 of Regulation (EC) No 1107/2009. In January 2023, the Court of Justice of the EU (CJEU) ruled in case C-162/21 that EU Member States were not permitted to use these emergency powers to authorize seed coating pesticides if already prohibited by a Commission Regulation. Therefore, all agricultural applications of neonicotinoids in the EU are now prohibited. Because imidacloprid is relatively persistent in the environment (Gautam and Dubey 2022), there is still a reasonable probability of residues of imidacloprid or other neonicotinoids being present in feed materials at low concentrations (Brühl et al. 2021, EFSA 2023). Furthermore, in most countries outside of the EU the use of neonicotinoids was either promoted (e.g., China) (Shao et al. 2013), or they are only subject to restrictions to limit exposure of pollinators (e.g., USA) (Klingelhöfer et al. 2022). Exposure of reared insects to these substances via contaminated feed materials is therefore not unlikely. Spinosad is permitted to be used as a plant protection product in regular agriculture (Regulation (EU) 2021/566) – but notably also in organic farming (Regulation (EC) No 889/2008, Annex II). This raises some questions on the presumed safety of organic produce for LMW rearing, if spinosad residues persist in the feed on which the insects are reared (Meijer et al. 2022a).

The objective of this study was to determine the potential effects of chronic exposure of *Alphitobius diaperinus* to sublethal concentrations of the insecticides imidacloprid and spinosad. To this end, larvae, pupae and adult beetles of two subsequent generations of this insect species were chronically exposed via the diet to spinosad and imidacloprid, at various concentrations, that were hypothesized to be lethal and sublethal to part of the population.

Materials and methods

Substrate preparation

The experimental treatments used in this experiment are shown in Table 1. Each of the two insecticidal substances of interest (spinosad, imidacloprid) were spiked to LMW substrate in two treatments: one concentration equal to the applicable MRL in wheat, and at 10 % of the MRL. The control treatment consisted of unspiked LMW substrate. Each treatment was performed in triplicate, except for the control which was performed in 6 replicates. All spiked substances used in this experiment were analytical reference materials for residue analysis. The supplier of all materials was Dr. Ehrenstorfer GmbH (Augsburg, Germany) and these were purchased from LGC Standards GmbH (Wesel, Germany). Spinosad was a mixture of spinosyn A and spinosyn D, present at 76.9 and 23.1 %, respectively. Methanol was used as a solvent for both substances.

Table 1: Overview of insecticidal substances in experimental treatments, including purity, CAS number and intended spiked concentration.

Treatment number	Substance name	Purity (%)	CAS Number	Spiked concentration (mg/kg)
1	Spinosad	94.0%	168316-95-8	2.0
2				0.2
3	Imidacloprid	98.55%	138261-41-3	0.1
4				0.01
5	Control	n/a	n/a	n/a

A total of at least 6 kg of spiked feed was prepared per treatment. The dry feed was provided by Research Diet Services, Wijk bij Duurstede, the Netherlands (same supplier as used by Meijer et al. (2022a)), and consisted of a dry mix of primarily organic wheat products, a vegetable protein source, and a pre-mix. All volumes and weights were doubled for the control treatment. Firstly, a slurry of dry feed and methanol was made. In order to minimize the occupational exposure to methanol, the total amount of feed was prepared in three separate batches of 2 kg each. This was also done to minimize any degradation of tested substances. A 'pre-mix' of 500 g of dry feed was mixed with approximately 0.7 l of methanol. The insecticide solutions were prepared such that the volume of the insecticide solutions equaled 0.5% of the used feed (2.5 ml to 500 g), to facilitate homogenous distribution of the added substance. The amount of active substance in each of these solutions was calculated to achieve the desired concentrations in 2.0 kg of total feed per batch, as shown in Table 1. All steps, except the addition of insecticidal substances, were also executed for the control treatment. Since methanol was used as a solvent for added substances as well as to make the slurry, the control treatment was effectively a solvent control. All slurried feed was placed in low, open aluminium containers in a fume hood for the methanol to evaporate. After 2 days, the dry feed was first loosely mixed with a metal spoon and deposited into a Stephan UMC 5 electronic Table-top mixer. From the high-concentration feed of each treatment, one 2.5 g aliquot ('pre-mix') was taken for analysis and stored at -18° C. Subsequently, 1.5 kg of blank feed was added and mixed with the spiked feed for approximately 2 min. Again, a 2.5 g aliquot was taken from each treatment ('post-mix'). The remaining feed was deposited into closed containers and stored at 7° C before the experiment. When the feed from each batch was used for the first time, a third 2.5 g aliquot was taken ('first feed'). Finally, when the last material from each batch was provided to the insects, two final 2.5 g aliquot sub-samples were taken from each treatment ('last feed'). These aliquots taken from each batch at different moments in time were analyzed to determine potential degradation of the active insecticidal substance in the feed throughout the experiment.

Experimental procedures

The bioassay design for this experiment is shown schematically in Figure 1. The experiment was performed at the premises of Ynsect NL (Ermelo, The Netherlands). On experimental day 1 (D1), exactly 400 mg of neonate larvae (provided by Ynsect NL)

were added to 12.0 g of substrate, consisting of 6.0 g of dry (spiked) feed and 6.0 ml added water. This was the designated parent (P1) generation. The insects were intermittently provided with the treated (spiked) feed throughout their entire lifecycle. The feeding schedule is shown in Table 2. On D25, the prepupae were sieved from the diet and frass. A representative sample of 3000 mg of larvae was taken from each replicate container and the number (n) of larvae per sample was counted twice, as described in Meijer et al. (2022a). If the discrepancy between the two counts was >1%, the sample was counted a third time: the mean value of the two closest counts was used to calculate the mean individual weight, which was extrapolated to the total yield to calculate the total number of larvae per replicate. Due to the non-invasive nature of this procedure, the larvae were placed back into the replicate container to pupate. The counting procedure was repeated on D53 with a sample of 3500 mg of beetles per replicate, which was counted in the same way as the larvae (twice or thrice) to determine the mean individual weight and the estimated number of beetles per replicate. The remaining larvae and pupae were also weighed, and subsequently discarded by freezing at -20° C. The beetles were placed in separate replicate containers. Starting from D54, every day, the eggs were removed from this replicate container: on days 4 and 5 after inoculation, the hatched first instar larvae were weighed. Assuming peak egg production around that time, the eggs of D67 and D68 were used to inoculate the experimental offspring generation F1. The day that these eggs hatched (D71 of P1 generation) was taken as D1 for the F1 generation. The same procedures as described for the P1 generation were followed for the F1 generation. Eclosion of first instar larvae from eggs produced by the beetles of the P1 generation was monitored until D124. On D25 of each generation, a 2.5 g larval sample was taken for subsequent chemical analysis to determine the concentration of the respectively spiked insecticide in the larvae of each treatment. The same was done for the beetles on D53. The larval samples of each treatment were pooled for chemical analysis.

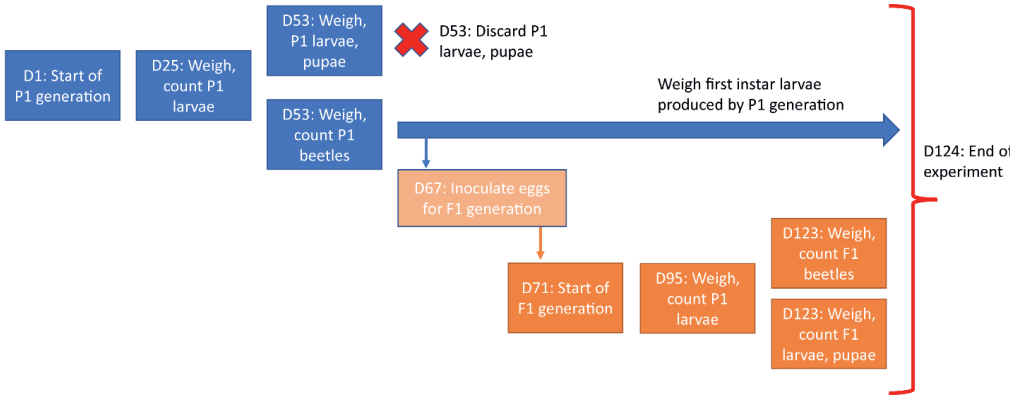

Figure 1: Schematic depiction of bioassay. Procedures performed on the parent (P1) generation are shown in blue; for the first offspring generation (F1) in orange. D represents the number of days since the start of the experiment.

Table 2: Feeding schedule: weight of prepared diet provided to insects for each life-stage.

Experimental day	**Generation and life-stage**	**Total weight of prepared (spiked) diet provided (g)**
D01-25	P1 Larvae	275
D26-48	P1 Pupae, beetles	160
D49-64	P1 beetles	600
D64-88	F1 Larvae	275
D89-111	F1 Pupae, beetles	160

Chemical analyses

Chemical analysis of samples was done with liquid chromatography-mass spectrometry (LC/MS-MS), in the same manner as described in Meijer et al. (2022a) and Meijer et al. (2021). In summary, extraction of assayed active substances was performed on 1.0 ± 0.05 g of frozen larval samples. These were diluted (2 ml of Milli-Q water (Millipore Sigma, Burlington, MA, USA) and 2 ml of acetonitrile (ActuAll Chemicals, Oss, The Netherlands) + 1% acetic acid (Merck, Darmstadt, Germany) and homogenized using an ultra-turrax machine (IKA Werke, Staufen, Germany), followed by addition of 0.5 g of sodium acetate (Merck) and 2 g $MgSO_4$ (VWR International, Amsterdam, The Netherlands), vortexing (Vortex 3, IKA Werke) for 30 s, and centrifugation (5 min at 3600 rpm, SL40R Thermo Scientific, Waltham, MA, USA) to induce phase separation; 250 µl of the acetonitrile phase was diluted 1:1 with Milli-Q water and filtered using an integrated filter vial (0.45 µm, PTFE, Cytiva, Marlborough, MA, USA). Analysis was performed on a Shimadzu (Kyoto, Japan) ultra-high-performance liquid chromatography (UPLC) system and an AB Sciex Qtrap 6500 MS (AB Sciex, Framingham, MA, USA). Details on LC and MS/MS conditions are provided in supplementary Table S2.

Of the three batches of feed prepared for this experiment, the second batch (randomly determined) was used to analyze the concentrations of spiked substances in all 5

aliquots per treatment. This was done to verify the spiked concentration and determine whether any degradation of spiked insecticides had taken place. For the first and third batch, only the aliquot taken directly after mixing and one aliquot taken at the end of the feed batch were analyzed to verify the spiked concentrations.

Data and statistical analysis

For the larvae on D25 of both generations, differences between treatments were tested using a Kruskal-Wallis test ($\alpha = 0.05$) for the variables yield, mean individual larval weight, and number of larvae. For variables for which differences were significant ($P \leq 0.05$), a post-hoc test (Mann-Whitney U test, $\alpha = 0.05$) was performed to compare each of the treatments ($n_1=3$) against the control ($n_2=6$). The same analyses were followed for these measures for beetles of the parent generation P1 on D53, as was done for the larvae, pupae and beetles of the offspring generation F1. For eclosion, the cumulative weight of first instar larvae produced by the parent generation P1 was analyzed in the same manner. Pupation was defined as the number of beetles for each replicate on D48 of a generation, as a percentage of the number of larvae on D25. Finally, differences between the control treatments of the two generations were compared using a Mann-Whitney U test ($\alpha = 0.05$). All statistical analyses were performed in SPSS Statistics for Microsoft Windows (version 25.0.0.2, IBM Corp., Armonk, NY, USA).

Results

Quality control

Analyzed concentrations of spiked substances in the feed are shown in Table S1. Overall, analyzed concentrations were in accordance with intended concentrations, i.e., deviations < 30 %. Recovery of certain samples exceeded the mentioned 30 % benchmark, however, adequate results for other samples of the same batch suggest that overall quality of the spiked feed was acceptable. For the lower spinosad concentration (0.2 mg/kg), recovery throughout all three batches was lower than intended. According to Hertlein et al. (2011), spinosad is stable in enclosed storage environments. This was also the case for the substrate treated in this experiment, as concluded from the analytical results of aliquots taken at different stages in this experiment giving no indication of degradation of the substance over time. As such, although the causes of lower concentrations than intended remain unclear; experimental results can be interpreted for concentrations as analyzed.

Insect performance

Larvae

For all performance variables, except the mean individual larval weight of F1, differences between the treatments were statistically significant, for both generations ($P \leq 0.05$; Figure 2 and Table S3). The post-hoc Mann-Whitney U test showed a significant decline in each of these three performance indicators for the treatment containing spinosad at 2.0 mg/kg in each of the two generations, compared to the controls ($P \leq 0.05$). For the P1 larvae exposed to the highest concentration of

imidacloprid (0.1 mg/kg), also a slight but significant reduction in yield and mean individual weight was observed; while higher values were found in the treatment with the lower concentration (0.01 mg/kg) of that substance ($P \leq 0.05$). A tendency towards an increase in number of F1 larvae was also observed for the lower concentration of spinosad (0.2 mg/kg) ($P \leq 0.05$).

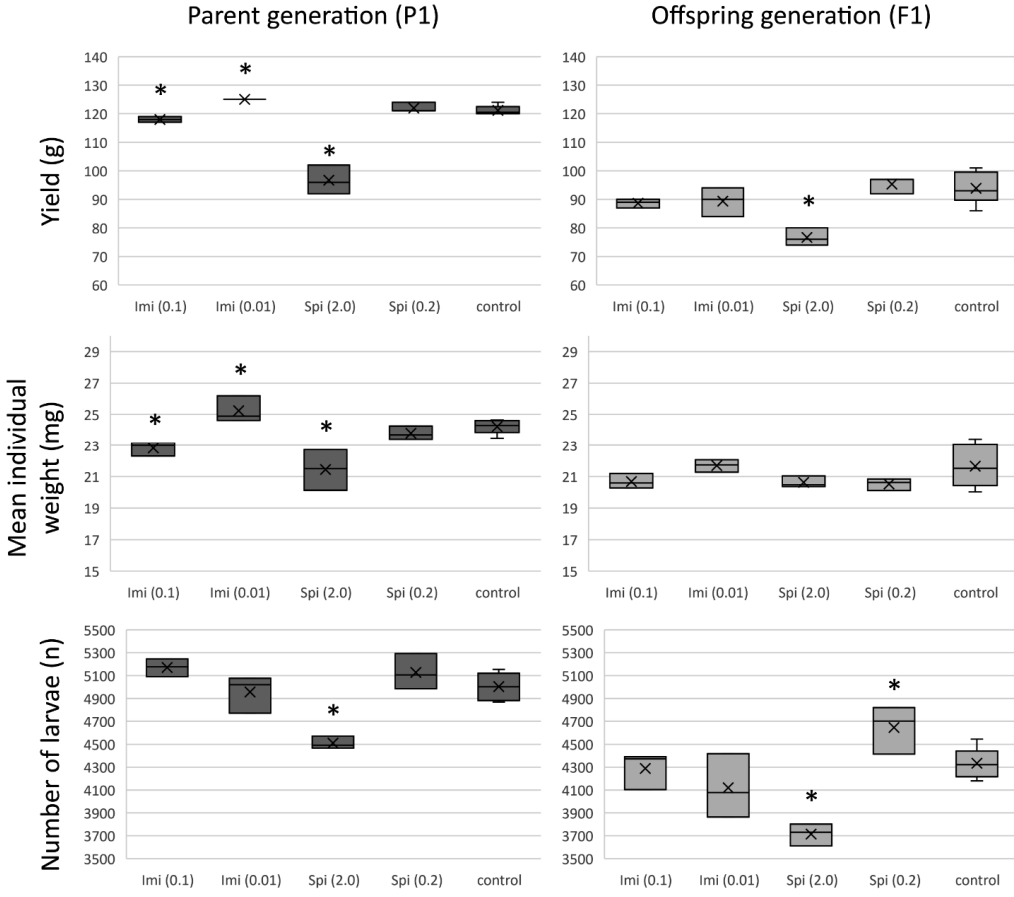

Figure 2: Yield (g), mean individual weight (mg), and number of individual larvae (n) of the first (P1, left) and second generation (F1, right). Mean and standard deviation of n=3 replicates for treatments and n=6 for the control. Asterisks (*) denote significant differences between a treatment and the control (Mann Whitney U test, α=0.05). Numerical data are shown in Table S3.

Adult beetles

For the beetles of parent generation P1, there were no significant differences between the treatments for any of the three tested performance variables yield, mean individual beetle weight, and number of beetles alive on experimental D48 ($P > 0.05$; Figure 3 and Table S3). The same was true for the yield and number of F1 beetles alive ($P > 0.05$). However, the mean individual F1 beetle weight was significantly different across different treatments ($P \leq 0.05$): exposure to the highest concentration of imidacloprid

(0.1 mg/kg) resulted in a significant decline of individual beetle weight ($P \leq 0.05$), while the highest concentration of spinosad caused a significant increase ($P \leq 0.05$).

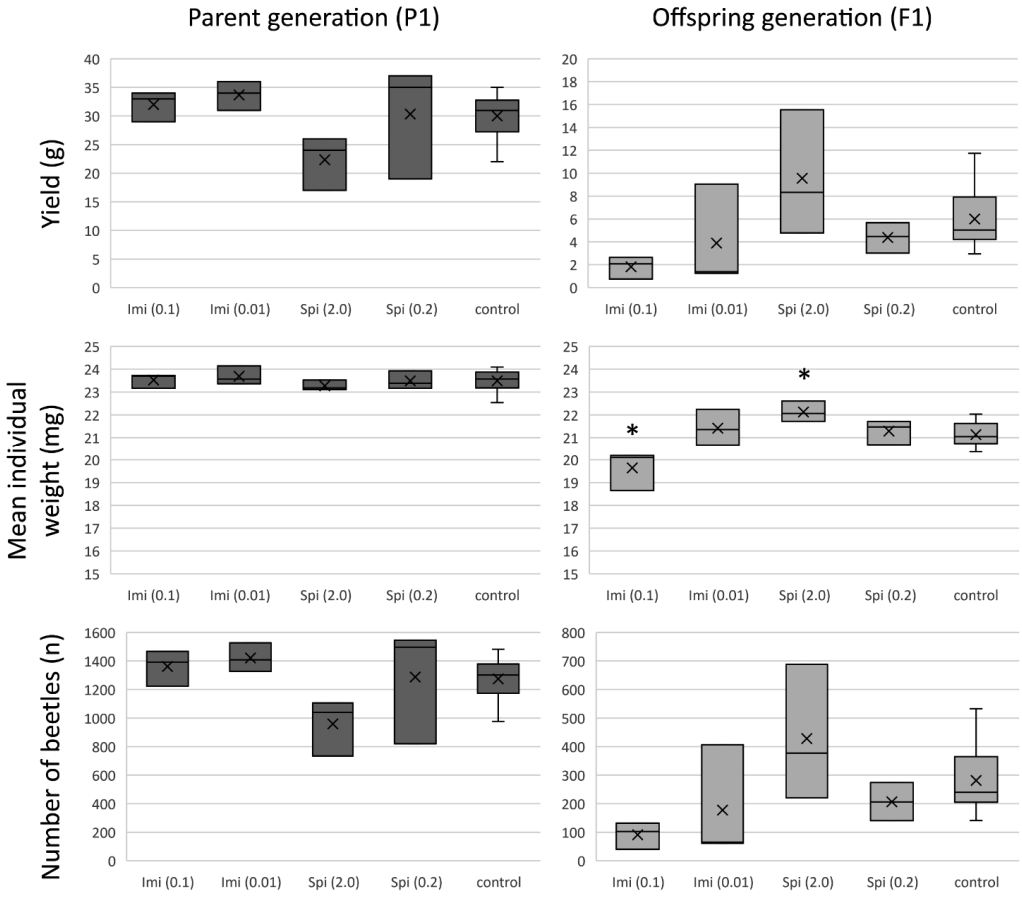

Figure 3: Yield (g), mean individual weight (mg), and number of beetles alive (n) of the first (P1, left) and second generation (F1, right). Mean and standard deviation of n=3 replicates for treatments and n=6 for the control. Asterisks (*) denote significant differences between a treatment and the control (Mann Whitney U test, α=0.05). Numerical data are shown in Table S3.

Pupation

The measure pupation was expressed as the number of beetles on D48 as a percentage of the number of larvae in that replicate on D25. For P1, only the total combined biomass of larvae and yet-to-emerge pupae on D48 was weighed; for F1, the yield and number of individuals of both these stages was determined separately. From Figure 4A, it is clear that inter-generational differences were substantial: in all treatments, pupation was higher for P1 than for F1. This difference can, to some extent, be explained by the comparatively higher combined biomass of F1 larvae and pupae, as shown in Figure 4B.

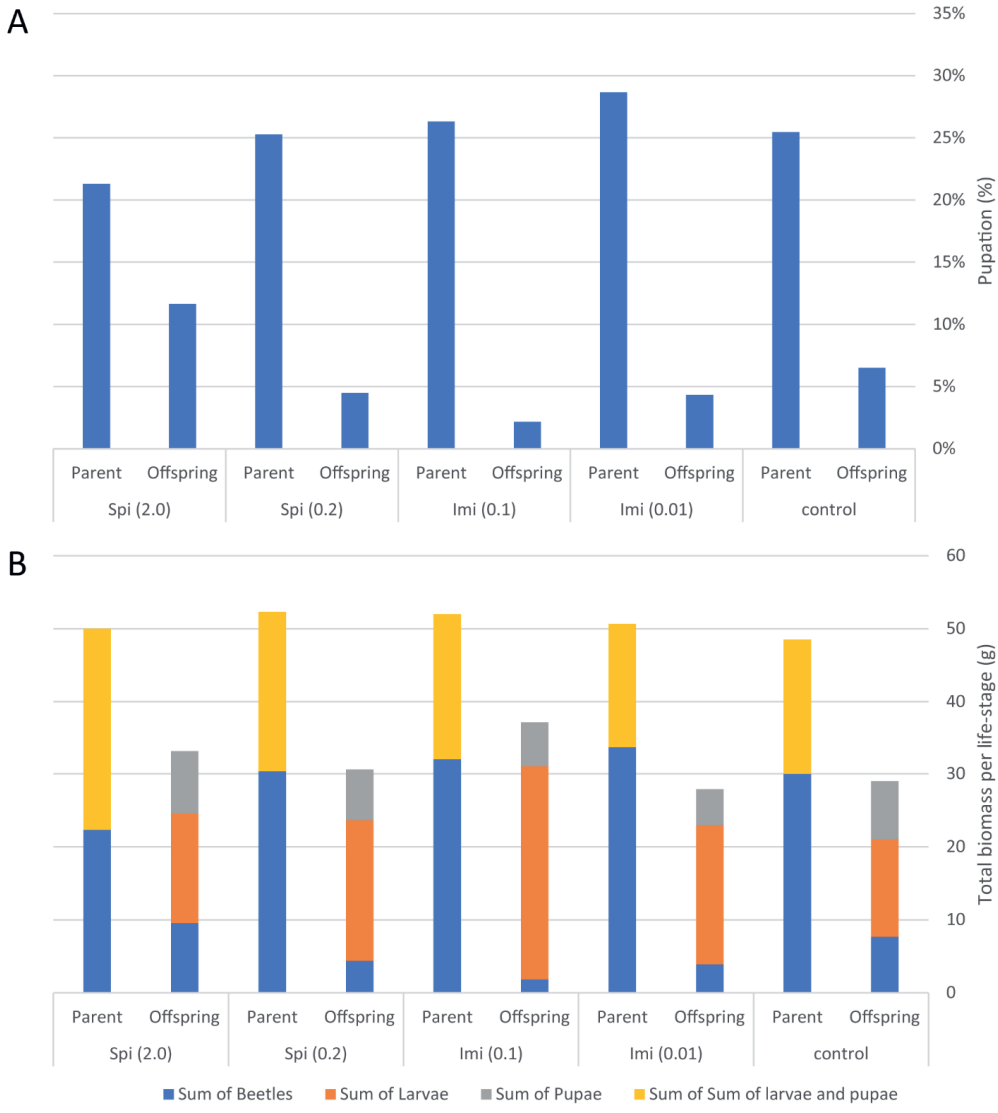

Figure 4A: Pupation for parent (P1) and offspring (F1) generations: the number of beetles on day 48 after eclosion as a percentage of the number of larvae on day 25. Figure 4B: Total biomass (g) per life-stage: beetles, larvae, and pupae for P1; beetles and sum of larvae and pupae for F1.

Eclosion

No significant differences in eclosion were observed between treatments ($P > 0.05$; Figure 5). For imidacloprid (0.1) the average value was approximately 70% of the average value of the control treatment.

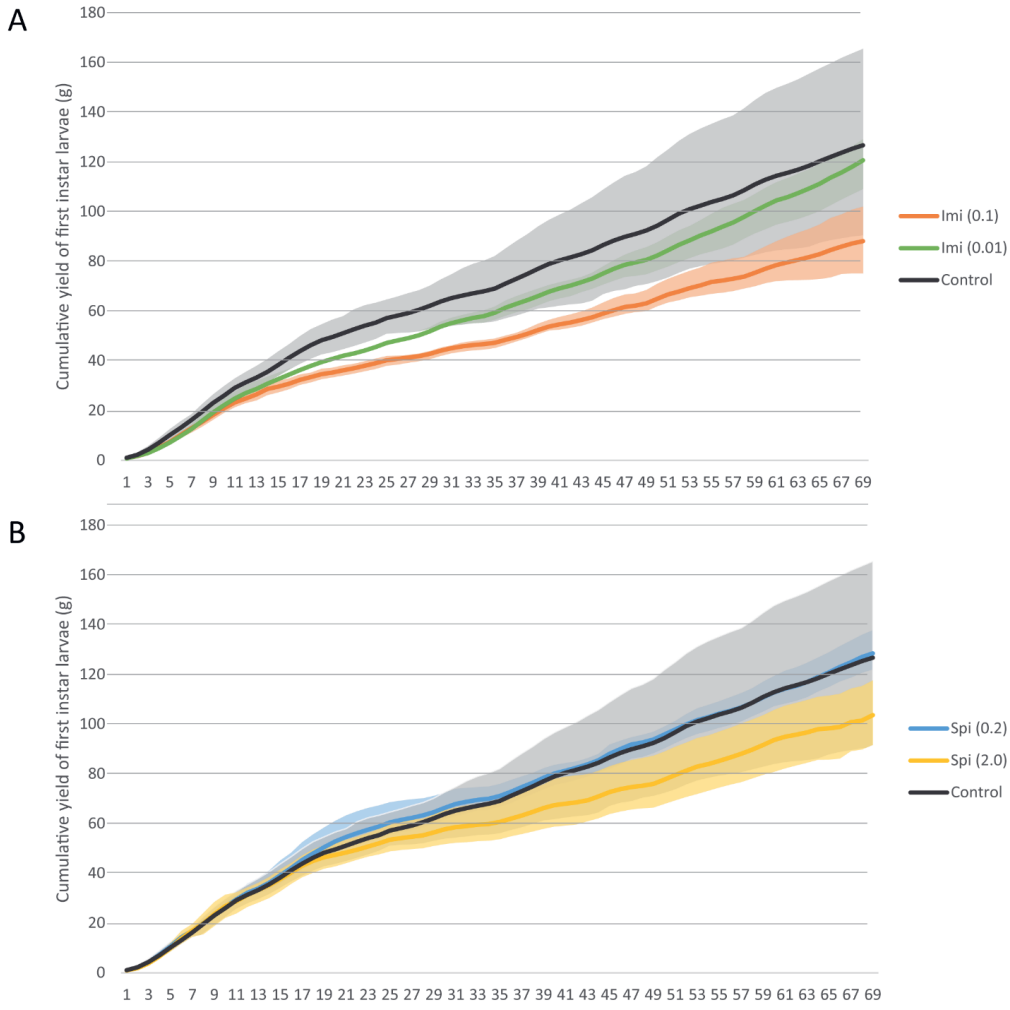

Figure 5: Stacked line chart of cumulative yield (g) of first instar larvae (after eclosion) per day per 100 g of parent beetles producing the F1 generation. Figure 5A shows data for imidacloprid at 0.1 and 0.01 mg/kg; Figure 5B shows data for spinosad at 0.2 and 2.0 mg/kg; both figures show the control data. Arithmetic mean for each treatment is shown as a line. The coloured areas between upper and lower data limits correspond to the colours of the lines. Overlap between areas is shown with a different colour.

Insecticide concentrations and transfer

Table 3 shows the concentrations in the LMW larvae against the concentrations in the feed, and the transfer of parent insecticides from the substrate into the larvae during the experiment. For all treatments, transfer of tested substances was below 10%. Comparing the transfer percentages between the two generations shows that these are approximately twice as high for F1 than for P1.

Table 3: Analysed concentrations (mg/kg) of spiked substances imidacloprid and spinosad in samples of feed and larvae. Transfer is expressed as a percentage of the analysed concentration in the larvae (pooled sample)divided by the analysed concentration in the feed (mean of samples taken at moments of first and last feeding from batch 1 (for P1) and 2 (for F1)).

Substance and intended concentration in feed (mg/kg)	**Analysed concentration feed (mg/kg)**	**Analysed concentration larvae (mg/kg)**	**Transfer (%)**
Generation 1 (P1)			
Imidacloprid (0.1)	0.094	0.001	1.5%
Imidacloprid (0.01)	0.020	0.001	3.1%
Spinosad (2.0)	1.855	0.006	0.3%
Spinosad (0.2)	0.141	0.000	0.1%
Generation 2 (F1)			
Imidacloprid (0.1)	0.082	0.003	3.2%
Imidacloprid (0.01)	0.011	0.001	6.9%
Spinosad (2.0)	1.993	0.010	0.5%
Spinosad (0.2)	0.120	0.001	0.6%

Discussion

Results of this study on direct lethal and sub-lethal effects of spinosad at a concentration of 2.0 mg/kg showed significant reductions in total larval yield and number of alive individuals as compared to the control for both the parent and offspring generation. The mean reductions in yield for both LMW generations were comparable to the reduction observed for spinosad in a previous study on *Alphitobius diaperinus* (-20 %; Meijer et al. 2022), which suggests that larval yields of the offspring were not affected by parental exposure. This is line with the suggested use of combining spinosad with an insect growth regulator (IGR), such as methoprene, to suppress reproduction, as an effective method for complete pest control (Athanassiou et al. 2011). In addition, spinosad has been found to increase the susceptibility of resistant LMW to synthetic pyrethroids (Lambkin and Furlong 2014), as was the case for permethrin, azadirachtin, and *Bacillus thuringiensis* toxin for the Colorado potato beetle (*Leptinotarsa decemlineata* (Say); Coleoptera: Chrysomelidae) (Igrc Barčić et al. 2006, Bažok et al. 2008). In this study, exposure to the lower concentration of spinosad (0.2 mg/kg) had little to no effect on any of the measured performance variables of either assayed generation. Nevertheless, the presence of multiple insecticide residues ('cocktails') in (compound) feed materials is estimated to be reasonably likely since the application of combined or rotated treatments are generally recommended principles of insecticide resistance management (IRM) (Rajendran 2020, Sparks et al. 2020). This implies commercial reared insects are likely to be exposed to residues of multiple insecticides. Further, since insecticidal

substances may have joint or synergistic action at lower concentrations than each substance individually (Hewlett and Plackett 1952) - as indicated above for spinosad in conjunction with methoprene or synthetic pyrethroids - the presence of spinosad in feed at lower concentrations (~0.2 mg/kg) may not be inherently safe for optimal rearing performance of LMW – if other insecticidal substances are also present.

Whereas the mean reduction in yield for imidacloprid at 0.1 mg/kg observed by Meijer et al. (2022a) was approximately -32 %, it was -3 % for the P1 generation in this study, and not significantly different from the control for the F1 offspring generation. The experiment by Meijer et al. (2022a) was executed with the same LMW population and at the same premises as the current study, but three years earlier. We therefore speculate that this particular population of *Alphitobius diaperinus* may have become more resistant to the toxic effects of imidacloprid or other neonicotinoids in the years since the execution of the previous experiment. Development of resistance to both spinosad and imidacloprid has been reported in different species of Coleoptera (Olson et al. 2000, Zhao et al. 2000, Mota-Sanchez et al. 2006). Mean eclosion in the treatment containing imidacloprid at 0.1 mg/kg was 70% of the mean control value, but this difference was not statistically significant, which is attributed to the large variation (min-max) in control values. Recommendations on alterations in experimental design of follow-up studies, to mitigate this issue of high variation in control values, are provided at the end of this section.

The offspring generation F1 performed considerably worse as compared to the parent generation P1: significant differences were observed between the performance of the two generations, in terms of all three performance variables (yield, individual weight, number of individuals alive, proportion pupation). This was the case for the various insecticidal treatments, as well as the controls. Pupation was higher for P1 than for F1, but this was partly offset by the higher combined biomass of F1 larvae and pupae, suggesting that F1 pupation and emergence were merely delayed as compared to P1. We speculate that these differences were due to the quality of the feed and, unfortunately, its low suitability for rearing *Alphitobius diaperinus* for reproduction. The particular feed used in this study was of organic quality to avoid any inherent insecticide residues, and the composition was chosen to be the same as the substrate used by Meijer et al. (2022a). For future studies using a similar methodology, we recommend that the *A. diaperinus* generation that is used to produce the experimental parent generation P1 are also reared on the control feed used in the study, to reduce any intra-generational effects of this potential substrate-related variable.

Accumulation of insecticidal compounds in the insect biomass, as a result of chronic and/or parental exposure, could present a food or feed safety issue. For all tested substances, concentrations in the larvae were doubled in the offspring generation F1 compared to the parent generation P1. This finding implies that *Alphitobius diaperinus* is less capable of metabolizing the imidacloprid and spinosad after prolonged chronic exposure. Nonetheless, all larval concentrations were below the MRL applicable to

invertebrate terrestrial animals as laid down in Regulation (EC) No 396/2005, for both imidacloprid (0.01 mg/kg) and spinosad (0.02 mg/kg). This suggests that the food safety issue of chronic exposure of *Alphitobius diaperinus* to these two insecticides (over the tested period of time of two generations) is minimal. It must be emphasized that these results are limited to the substances spinosad and imidacloprid; transfer rates for other insecticidal substances to LMW larvae could be different. The MRLs applicable to insects, being invertebrate terrestrial animals, are set at the substance-specific defaults ranging from 0.01 to 0.05 mg/kg. Any transfer or accumulation resulting in exceedance of those limits would make the insect food or feed product uncompliant with Regulation (EC) No 396/2005. However, published literature on experimental transfer of insecticidal substances from substrate to biomass of LMW (Meijer et al. 2022a), or other reared insects (Dreassi et al. 2020, Meijer et al. 2021), is severely limited: more research is therefore needed.

The bioassay used in this study was in essence an adapted extended one-generation reproductive toxicity experiment (OECD 2018). The study execution was resource- and time-intensive (127 days in total), particularly when compared to an experiment focusing only on the larval stage of *Alphitobius diaperinus*, which entails approximately 20-25 days until harvest (Meijer et al. 2022a, Meijer et al. 2022b). As such, we recommend that alternative bioassay methods are developed to determine their use in testing for sub-lethal effects on insect species reared for food or feed. This recommendation also applies to other insect species used for food and feed purposes, such as *Tenebrio molitor* with a larval cycle of at least 57 days in controlled conditions (Ribeiro et al. 2018). Alternative bioassays exploring sub-lethal effects have employed cameras and software to assess the (larval) motility response to insecticidal exposure (Tooming et al. 2014, Denecke et al. 2015). To our knowledge, no such bioassays have been developed for LMW or other reared insect species to date. Additional research is pertinent due to the risk of adverse effects on the reproductive potential of reared insect populations even in case of exposure to low concentrations – for instance in case of multiple insecticides, as discussed above – which could present a major financial burden for insect rearing companies. Much recent research has focused on sub-lethal effects of insecticides, particularly neonicotinoids such as imidacloprid, on honey bees in relation to Colony Collapse Disorder (Wu-Smart and Spivak 2016, De Smet et al. 2017, Chambers et al. 2019), but the application of those experimental designs to reared insects such as LMW is questionable due to the highly differing conditions. Exploratory results from experiments focusing on one or two subsequent life-stages could subsequently be validated for the determination of chronic exposure during commercial rearing conditions in an extended one-generation (OECD 2018), or two-generation (OECD 2001) reproductive toxicity experiment.

Conclusion and recommendations

The objective of this study was to determine the potential effects of chronic exposure of two subsequent generations of *Alphitobius diaperinus* to sublethal concentrations of

the insecticides imidacloprid and spinosad. Effects on total biomass yield, individual insect weight, and survival were determined, as well as possible transfer of the insecticide to the larvae. Results showed significant adverse effects of spinosad at a concentration of 2.0 mg/kg on most performance variables of the larvae of both generations. For the parent, but not the offspring generation, imidacloprid also caused reductions in yield and mean individual larval weight at the higher concentration of 0.1 mg/kg as compared to the control but an increase for those measures at the lower tested concentration (0.01 mg/kg). Direct adverse effects on beetles were only observed for imidacloprid at 0.1 mg/kg, but eclosion data for this treatment implied that production of offspring was negatively affected. Concentrations of the two tested substances in larval samples of both generations did not give cause for food safety concern. Given the many different insecticides used in agriculture, more research is needed to investigate the potential of a variety of insecticide residues to induce sub-lethal effects to reared insect populations of *A. diaperinus*, as well as other species that are being reared for food and feed purposes. Also, sub-lethal effects from insect exposure to a cocktail of insecticides needs to be investigated. A focus in future research on insect growth regulators is recommended. The resources required to determine sub-lethal effects on multiple generations of *A. diaperinus* as a result of chronic dietary exposure are concluded to be prohibitive for initial assessments, and development of more cost-efficient bioassay designs is therefore needed.

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Supplementary materials

Table S1: Concentrations (mg/kg) of spiked substances per treatment in samples collected at different time points per batch. Recovery expressed as a percentage of the analysed concentration divided by the intended concentration. For spinosad, the concentration is the sum of spinosyn A and D, shown in brackets.

Spiked insecticidal substance	**Sample ***	**Intended concentration (mg/kg)**	**Analysed concentration (mg/kg)**	**Recovery (%)**
Batch 1				
Imidacloprid	Post-mix	0.10	0.01	95
	Last feed	0.10	0.01	92
Imidacloprid	Post-mix	0.01	0.02	186
	Last feed	0.01	0.02	206
Spinosad (spinosyn A/D)	Post-mix	2.00	1.92 (1.49 / 0.43)	96
	Last feed	2.00	1.79 (1.40 / 0.39)	89
Spinosad (spinosyn A/D)	Post-mix	0.20	0.14 (0.11 / 0.03)	71
	Last feed	0.20	0.14 (0.11 / 0.03)	70
Batch 2				
Imidacloprid	Pre-mix	0.40	0.38	95
	Post-mix	0.10	0.10	96
	First feed	0.10	0.10	101
	Last feed A	0.10	0.10	102
	Last feed B	0.10	0.10	97
Imidacloprid	Pre-mix	0.04	0.04	96
	Post-mix	0.01	0.01	90
	First feed	0.01	0.02	160
	Last feed A	0.01	0.01	107
	Last feed B	0.01	0.01	114
Spinosad (spinosyn A/D)	Pre-mix	8.00	6.77 (5.11 / 1.66)	85
	Post-mix	2.00	2.18 (1.64 / 0.54)	109
	First feed	2.00	1.68 (1.31 / 0.36)	84
	Last feed A	2.00	2.29 (1.73 / 0.56)	115
	Last feed B	2.00	2.25 (1.71 / 0.55)	113
Spinosad (spinosyn A/D)	Post-mix	0.20	0.17 (0.13 / 0.04)	87
	First feed	0.20	0.15 (0.12 / 0.03)	77
	Last feed A	0.20	0.21 (0.16/ 0.05)	104
	Last feed B	0.20	0.13 (0.11 / 0.03)	67
Batch 3				
Imidacloprid	Post-mix	0.10	0.09	85
	Last feed	0.10	0.08	78
Imidacloprid	Post-mix	0.01	0.01	114
	Last feed	0.01	0.01	107
Spinosad (spinosyn A/D)	Post-mix	2.00	1.82 (1.37 / 0.47)	91
	Last feed	2.00	2.17 (1.632 / 0.53)	108
Spinosad (spinosyn A/D)	Post-mix	0.20	0.12 (0.10 / 0.03)	61
	Last feed	0.20	0.12 (0.09 / 0.03)	59

Pre-mix: sample taken after evaporation of solvent used for slurry, before mixing with control feed;
Post-mix: sample taken after mixing with control feed;
First feed: sample taken at time when first feed of batch was provided to insects;
Last feed: sample taken at time when last feed of batch was provided to insects.

Table S2: Details on LC and MS/MS conditions

UPLC Gradient conditions for all tested substances.					
Time (min)		**Flow (ml/min)**	**A%**	**B%**	
-		0.4	100	0	
1		0.4	100	0	
6		0.4	0	100	
9		0.4	0	100	
9.5		0.4	100	0	
12		0.4	100	0	
MS/MS conditions					
Q1	**Q3**	**Substance ID**	**DP**	**CE**	**CXP**
732	142	Spinosyn A	186	41	20
732	98	Spinosyn A 2	186	93	22
746	142	Spinosyn D	186	43	20
746	99	Spinosyn D 2	186	75	12
256.1	175.1	Imidacloprid	41	25	12
256.1	209.1	Imidacloprid 2	41	23	14

Eluent A: Water with 5 mM ammonium formate and 0.1% formic acid
Eluent B: Methanol:water 95:5, with 5 mM ammonium formate and 0.1% formic acid
Gradient: see table
Injection volume: 5 µl
Column temperature: 40°C

Table S3: Overview of performance of P1 (parent) and F1 (offspring) generations against the insecticidal treatments containing imidacloprid (Imi) at 0.1 and 0.01 mg/kg, and Spinosad (Spi) at 2.0 and 0.2 mg/kg. On day 25 since eclosion for larvae in terms of yield, mean individual larval weight, and number of individuals alive. On day 48 after eclosion for beetles, and larvae and pupae combined, in terms of yield, mean individual larval weight, and number of individuals alive. Arithmetic mean and standard deviation of n=3 replicates for treatments, and n=6 for the control.

Stage	**Measure**	**Gen.**	**Imi (0.1)**	**Imi (0.01)**	**Spi (2.0)**	**Spi (0.2)**	**Control**
25 days after eclosion							
Larvae	Yield (g)	P1	118.0 ± 1.0	125.0 ± 0,0	96.7 ± 5.0	122.0 ± 1.7	121.2 ± 1.6
		F1	88.7 ± 1.5	89.3 ± 5.0	76.7 ± 3.1	95.3 ± 2.9	93.8 ± 5.5
	Mean ind. Weight (mg)	P1	22.8 ± 0.5	25.2 ± 0.8	21.4 ± 1.3	23.8 ± 0.4	24.2 ± 0.5
		F1	20.7 ± 0.5	21.7 ± 0.4	20.6 ± 0.4	20.5 ± 0.4	21.7 ± 1.3
	Number (n)	P1	5171 ± 77	4956 ± 162	4510 ± 55	5128 ± 155	5003 ± 118
		F1	4289 ± 160	4120 279	3715 ±97	4646 ± 210	4334 ± 132
48 days after eclosion							
Beetles	Yield (g)	P1	32.0 ± 2.6	33.7 ± 2.5	22.3 ± 4.7	30.3 ± 9.9	30.0 ± 4.4
		F1	1.8 ± 1.0	3.9 ± 4.5	9.6 ± 5.5	4.4 ± 1.3	7.7 ± 3.7
	Mean ind. Weight (mg)	P1	23.5 ± 0.3	23.7 ± 0.4	23.3 ± 0.2	23.5 ± 0.4	23.5 ± 0.5
		F1	19.6 ± 0.9	21.4 ± 0.8	22.1 ± 0.4	21.3 ±0.5	21.1 ± 0.6
	Number (n)	P1	1361 ± 125	1421 ± 101	959 ± 198	1288 ± 406	1275 ± 168
		F1	91 ± 47	178 ± 198	428 ±238	207 ±67	282 ± 134
	Pupation (%)	P1	26.3 ± 2.0	28.7 ± 1.5	21.3 ± 4.6	25.3 ± 8.5	25.5 ± 3.2
		F1	2.2 ± 1.2	4.3 ± 4.9	11.6 ± 6.8	4.5 ± 1.6	6.5 ± 3.2
Larvae	Yield (g)	F1	29.2 ± 5.0	19.0 ± 5.3	15.0 ± 7.6	19.3 ± 13.3	13.3 ± 2.8
	Mean ind. Weight (mg)	F1	32.5 ± 1.2	33.7 ± 1.5	36.3 ± 0.4	34.9 ± 0.7	34.5 ± 0.8
	Number (n)	F1	901.5 ± 171.1	569.6 ± 177.1	414.0 ± 209.7	552.9 ± 36.7	491.4 ± 145.5
Pupae	Yield (g)	F1	6.1 ± 1.5	5.0 ± 0.7	8.6 ± 1.5	6.9 ± 1.2	8.0 ± 1.5
	Mean ind. Weight (mg)	F1	25.7 ± 1.3	27.3 ± 1.8	27.6 ± 1.0	28.1 ± 1.8	27.6 ± 1.7
	Number (n)	F1	236.9 ± 50.8	183.9 ± 39.2	311.7 ± 62.6	247.4 ± 41.6	301.8 ± 52.9
Larvae + pupae	Yield (g)	P1	20.0 ± 2.6	17.0 ± 1.0	27.7 ± 7.2	22.0 ± 7.2	18.5 ± 2.3
		F1	35.4 ± 3.8	24.0 ± 5.8	23.6 ± 8.7	26.2 ± 0.3	25.2 ± 5.3
	Mean ind. Weight (mg)	P1	-	-	-	-	-
		F1	31.1 ± 1.1	32.1 ± 1.5	32.4 ± 0.6	32.8 ± 0.9	31.9 ± 0.4
	Number (n)	P1	-	-	-	-	-
		F1	1138 ± 145	753 ± 208	726 ± 263	800 ± 25	793 ± 177

-: not measured for this generation.

Chapter 6

General discussion

(Laboratory technician performing extraction for analysis. Photo by author.)

Introduction

The global population is expected to grow to 9.8 billion people in 2050 which will require the production of 60% more food. At the same time, immense pressure is placed on natural systems due to deforestation, soil degradation, depletion of water, loss of biodiversity, and of course climate change (Hossain et al., 2020; Kopittke et al., 2019; McLaughlin & Kinzelbach, 2015). Society tends to blame industry and agriculture as the main causes of the pressure on environmental resources. Agriculture is seen as one of the most important sectors that will be bearing negative consequences of climate change, as well as being part of the solution. Major shifts towards more sustainable forms of agricultural production are therefore paramount to meeting society's demands and ensuring a liveable planet for future generations (Wiebe et al., 2019; Wijerathna-Yapa & Pathirana, 2022). One such shift is the need to move from the production and consumption of meat, especially beef, to more sustainable options: due to the substantial environmental impacts of meat production. Proteins of vegetable, microbial, and insect origin are being considered as the most likely meat alternatives, although full dietary replacement of meat with these alternative protein sources seems unlikely (Fasolin et al., 2019; Munialo et al., 2022; Niva & Vainio, 2021; Siegrist & Hartmann, 2023).

The use of insects as food and feed could be one of the key steps towards meeting a variety of sustainability objectives (Van Huis & Oonincx, 2017; Veldkamp et al., 2022). Most importantly, insects can be a key link in a more circular food system, due to their ability to be reared on organic waste that would be unsuitable for other farmed animals (Cadinu et al., 2020; Van Zanten et al., 2015; Wade & Hoelle, 2020). Entomophagy, the practice of eating insects, has a long history in Africa, Asia, and South America, but not, or only scantly, in the cultural history of Europe and North America (Dobermann et al., 2017; Payne et al., 2019; Raheem et al., 2019; Yen, 2009). Multiple factors play a role in the acceptance of consumption of insects by humans, or lack thereof (Kauppi et al., 2019; Van Thielen et al., 2019). For the use of insects as animal feed in the European Union (EU), it are mainly historical legal barriers that have blocked opportunities (Bosch et al., 2019; Lähteenmäki-Uutela et al., 2021). The EU had faced multiple public health crises in the late 1990s and early 2000s due to diseases that originated from the recycling of various materials of animal origin for those same animals (i.e., cannibalism). Examples of such diseases and related crises include bovine spongiform encephalopathy (BSE) and the resulting variant Creutzfeldt–Jakob disease (vCJD) in humans, as well as animal diseases such as foot-and-mouth disease, and swine fever. The drastic measures that were needed to curtail the proliferation of these diseases still echo in the current legal landscape for animal feed and food safety. Specifically, the feeding of ruminants with feed of animal origin became prohibited (feed ban) and this ban was later extended to other farmed animals as well (extended feed ban), with few exceptions such as milk and eggs. Insects were not specifically exempted, and the mentioned feed bans therefore implicitly prohibited their inclusion in animal feed. Due to the major consequences from these historical public health crises

and the fear of repetition, the precautionary approach to adoption of new exemptions has been accordingly slow (Van Raamsdonk et al., 2007; Van Raamsdonk et al., 2017). Not until 2017 was the feeding of 'processed animal proteins' (PAPs) from specified insect species to aquaculture animals permitted (Regulation (EU) No 2017/893), and only in 2021 was this permission also extended to pigs and poultry (Regulation (EU) 2021/1372). Although insects may now at least be fed to such 'conventional' livestock animals, the substrates on which the insects may be reared are still largely limited to materials of plant origin, thereby inefficiently competing for feed with those same animals to which the insects are intended to be fed (Meijer et al., 2023; Van Raamsdonk et al., 2023). Until substrate options for reared insects are expanded to sources with lower or no competition for feeding to conventional production animals, the potential of insects to contribute to the reduction of the environmental impact of the agricultural and food sector cannot be fully realized (Jucker et al., 2020).

One of the main pressures that agriculture exerts on natural systems is the use of insecticides against 'pest' insect species in arable farming, but through this, also the killing of many non-target animals. In 1962, Carson discussed the effects of the wide-spread use of insecticides in the United States to exterminate certain insects such as the fire ant, but thereby also inadvertently killing the songbirds and other non-insect wildlife, thus predicted to cause a 'Silent Spring' (Carson, 1962). The publication of this book caused the primary offenders dichlorodiphenyltrichloroethane (DDT), as well as other organochlorines such as aldrin and dieldrin, to be banned in most parts of the world, but new pesticides took their mantle and the global catastrophe that is facing insects is greater than ever (Davis, 2019; Goulson, 2021; Milman, 2022). Alarming declines in insect abundance, diversity, and biomass have been reported and continued agricultural use of insecticides is a major culprit (Forister et al., 2019; Hochkirch, 2016). Examples of new insecticidal substances that have been developed since the ban on organochlorines include compounds in the classes of organophosphates, carbamates, pyrethroids, and neonicotinoids (Epstein, 2014).

Directive 2009/128/EC established a framework for Community action to achieve the sustainable use of pesticides, which required Member States to implement a variety of systems for improving training of professional users, requirements on sales, facilitating research, and public awareness campaigns, among other aspects. In addition, the European Commission (EC) services published a database ('toolbox') presenting an overview of currently available 'integrated pest management' (IPM) methods (DG AGRI, 2023, n.d.). Most recently, the EC formulated a goal of a 50% reduction in pesticide use by 2030 in its Green Deal (Alexoaei et al., 2022; EC, 2022a; Heyen et al., 2020; Silva et al., 2022). However, such a reduction is expected to result in lower yields and higher prices for some agricultural products (Bremmer et al., 2021), and the plan thus received pushback from industrial and agricultural groups, as well as Member States (Howard & Boren, 2020; Wax, 2022). Overall, sales (and presumed use) of insecticides have been relatively stable over the years: although the trend is downward, it is slow, and the Green Deal target of -50% by 2030 is not in reach (EC, 2022b; Eurostat, 2023).

The continued agricultural use of insecticides, and thus their presence in substrates of reared insects, therefore seems likely.

The primary objectives of the research presented in this thesis were to assess the effects of insecticide residues present in the feed substrate on reared insects, taking into account the effects of such substances on growth and survival of exposed insects, as well as substance transfer to the insect biomass to be used for food or feed purposes. As focal species black soldier fly larvae (BSFL, *Hermetia illucens* (L.); Diptera: Stratiomyidae) and lesser mealworm (LMW, *Alphitobius diaperinus* (Panzer); Coleoptera: Tenebrionidae) were selected. Because the legal limits for insecticide residues in feed materials used to rear insects on are set in accordance with the ALARA principle (As Low As Reasonably Achievable), doses (concentrations) used in the experiments were in line with this principle. Prior to the research presented in this thesis, it was hypothesized that direct mortality resulting from exposure to such low concentrations would be low, but that sub-lethal reductions in biomass yield as well as certain amplifying factors such as joint effects caused by the presence of multiple substances in the substrate could still cause considerable losses. Specific attention in the experimental designs was therefore paid to the effects of multiple residues to which exposure occurs simultaneously ('cocktail' effects) and to sub-lethal effects on population upkeep (i.e., reproduction).

In this final Chapter, I will discuss the results from the four preceding Chapters in relation to one another and in the context of the most recent relevant scientific literature. The outline of this Chapter is as follows. The main findings of each Chapter are summarized in section 2. Secondly, the methodological developments of the experimental research presented in this thesis are highlighted in section 3. This is followed by an overview and synthesis of the analysed concentrations of tested substances in the insect biomass in section 4. In section 5, the experimental results are reviewed through a lens of legal implications. Finally, specific recommendations for industrial, policy, and academic stakeholders are provided in section 6, together with a future outlook on the European insect rearing industry.

Main findings

In Chapter 2 of this thesis, BSFL were exposed to several insecticidal substances that each represented a particular Mode of Action (MoA). Substances tested were chlorpyrifos, propoxur, cypermethrin, imidacloprid, spinosad, tebufenozide, and piperonyl butoxide (PBO). PBO is not an insecticidal substance by itself, but rather a synergist generally used in conjunction with a pyrethroid such as cypermethrin. Therefore, in addition to the treatments containing the two substances in isolation, a treatment combining both the active substance and synergist was also included. The selection was based on incidence data on insecticides in feed products, so that the results would have direct relevance for insect rearing organizations. The BSFL were exposed via the artificially contaminated ('spiked') diet to concentrations of selected substances that were equal to the maximum residue levels (MLRs) of these substances

in maize, or feed in general. After execution of these bioassays, samples of the substrate, insects, and remaining residual biomass ('frass') were analysed to determine the concentrations in each matrix. Results suggested that, at tested levels, the BSFL were susceptible to spinosad and cypermethrin and significant reductions in yield were observed for these treatments. The adverse effects of cypermethrin were augmented by the presence of the synergist PBO. Imidacloprid, however, caused a significant gain in yield compared to the control. Chemical analyses showed minimal transfer of tested substances to the insect larvae and accordingly no bioaccumulation was found, and mass balance calculations suggested that most substances had in fact been metabolized to some extent.

In Chapter 3 of this thesis, LMW were assayed against the same substances tested in Chapter 2, in addition to fipronil and pirimiphos-methyl. This design allowed for a comparison of the effects of most of the tested substances between the two assayed species BSFL and LMW. It was observed that spinosad also adversely affected LMW yields, like seen with the BSFL (in Chapter 2) but that imidacloprid caused a significant reduction in LMW yield – in contrast to the increase seen in BSFL. All tested substances were either not, or barely quantifiable in the analysed insect samples, showing very limited transfer.

Based on the results of Chapter 2, in particular the observed amplifying effect of PBO on cypermethrin, the study design of Chapter 4 focused on investigating the joint action of multiple substances on BSFL. Effects on growth and survival – combined in the BSFL total yield measure – as well as possible transfer were studied. The study incorporated an elaborate design in four parts. Firstly, the BSFL were exposed to gradually increasing concentrations of a representative of the pyrethroid class, i.e. cypermethrin, and an organophosphate representative, pirimiphos-methyl. Based on experimental data for both substances, a concentration/response curve was modelled, employing the benchmark dose (BMD) approach to estimate the concentration at which 10% and 50% reductions in yield could be expected at exposure to each of the two insecticides. Secondly, the relative potency of PBO by addition to cypermethrin was determined. This was done by testing cypermethrin and PBO at different ratios (1:1, 1:10, 1:20), and subsequently exposing the BSFL to different treatments including both substances at a ratio of 1:20 to also determine a concentration/response curve for their joint action. The curves for the effects of cypermethrin in isolation and with PBO on BSFL yield were compared to one another to determine the relative potency of cypermethrin by the addition of PBO. Thirdly, the hypothesis that substances with the same MoA would have similar effects was tested. Two different pyrethroids (permethrin and deltamethrin) and two other organophosphates (chlorpyrifos-methyl and malathion) were tested at the critical effect dose resulting in minus 50% yield (CED_{50}) that had been calculated for the two respective representative compounds. In addition, the 'analogues' were tested at a third of the CED_{50} in isolation, and in treatments with all three substances in the same class combined. This design allowed for a comparative toxicity assessment of these six substances. Fourthly, post-exposure insect samples of the third experiment

were analysed to determine concentrations and transfer of tested substances from substrate to the insect biomass. These concentrations were compared against the substance-specific maximum residue limit (MRL) for insects.

Results from Chapter 4 suggested that cypermethrin was more toxic than pirimiphos-methyl, with CED_{10} values of 0.4 and 4.8 mg/kg, respectively. For cypermethrin and PBO combined, the CED_{10} was 0.2 mg/kg and the relative potency factor resulting from the addition of PBO was 2.6. Of the three tested pyrethroids, deltamethrin was highly toxic to BSFL: at a mere 0.5 mg/kg, a 94% reduction in yield was observed. The three organophosphates combined appeared to have additive effects. Finally, although none of the substances accumulated in the insects, some showed relatively high transfer rates which could result in non-compliance of insect products due to the low MRLs for insects. It was concluded that the combined presence of insecticide residues in insect substrate could be highly problematic for the insect rearing industry due to these significant additive and synergistic adverse effects at relatively low concentrations and the potential for substance transfer resulting in concentrations in the insects exceeding the MRL.

Finally, in Chapter 5, two generations of *A. diaperinus* were exposed to spinosad (2.0 and 0.2 mg/kg) and imidacloprid (0.1 mg/kg). These two substances were selected due to the significant adverse effects on yield, as observed in Chapter 3 of this thesis. The spiked concentrations were equal to the MRL, and a tenth thereof. The study objective was to determine whether these two substances could induce sub-lethal effects on the reproductive capacity of this species. To this end, *A. diaperinus* larvae and beetles of two subsequent generations were chronically exposed to spinosad and imidacloprid. Effects on performance of larvae and beetles, as well as on pupation and eclosion, were measured; and larval samples were analysed to determine transfer of spiked substances from substrate to insect biomass. Results showed significant negative effects of spinosad at 2.0 mg/kg on larval performance, and of imidacloprid on beetle performance at 0.1 mg/kg. Reduced adverse effects caused by imidacloprid, compared to the significant yield losses observed in Chapter 3, were hypothesised to be a result of this population having become more resistant to the toxic effects of this substance.

Improvement of methodology

As far as I am aware, the studies presented in this thesis form the first comprehensive body of work investigating the effects of insecticide residues specifically on insects reared for food and feed. Other studies have investigated certain effects of insecticidal substances in studies on BSFL (Purschke et al., 2017; Tomberlin et al., 2002) and yellow mealworm (YMW, *Tenebrio molitor* (L.) Coleoptera: Tenebrionidae) (Dreassi et al., 2020), but not in the subsequent manner described here, and not on LMW. This thesis has combined multiple fields of research, including food safety, toxicology, and entomology to develop novel insights which can also be applied for commercial insect rearing, insecticide toxicity, policy making, and science. Due to the explorative nature of the experimental work, there are some inherent limitations and assumptions which

may nuance the implications of certain findings. For instance, experiments were held at small commercial rearing scale and it is uncertain to what extent the results also apply at larger scale or to other commercially reared insect populations, since such differences have been observed in pest populations of, for instance, LMW (Hickmann et al., 2018; Renault & Colinet, 2021; Singh & Johnson, 2015) (see Chapter 3). Despite the limitations, the results of this thesis present the first clear evidence that insecticide residues in substrates of insects reared for food and feed purposes can have significant adverse effects on a variety of performance measures and, potentially, compliance of insects with legal safety limits for insecticides.

The research presented in Chapters 2 and 3 made very clear that sub-lethal effects such as changes in total biomass yield should be the primary measure of insecticidal effects on commercially reared insects, rather than the more traditional measure of mortality used in insecticide toxicity research. The term 'yield' is a function of the measures survival and mean individual larval weight, which are measures of lethality and sub-lethality, respectively. The measure of yield, i.e., total production volumes of batches of insects, is a crucial key performance indicator (KPI) for insect rearing enterprises. The results presented in this thesis show that reductions in yield, compared to the control treatments, were not reflected by observed mortality. The larvae of both assayed species became (much) smaller as a result of dietary exposure, lower total yield, but survival was often only slightly affected. It is worth noting that care has been taken to ensure that the total mass of exposed insects between replicates at the start of each experiment larvae was homogenous, to control for potential differences in individual larvae size affecting results. It cannot be excluded that the size distribution of the larval population within each replicate at the start of the experiment could have affected results: the smaller larvae dying or staying small from insecticidal exposure, while bigger larvae grew (even) bigger (Robertson et al., 2017). The considerable variation in substance-specific measured individual larval weight post-experiment, as presented in Chapter 4, substantiate this hypothesis. However, the number of larvae used per replicate was assumed to be representative for the population and any observed effects would therefore have direct application for commercial-sized rearing.

In addition to yield, Chapter 5 focused on a wide spectrum of other sub-lethal effects on LMW resulting from multi-generational and chronic exposure. As far as I am aware, this was the first study to specifically investigate such effects on multiple life-stages of an insect species in the context of commercial rearing. Results implied that such effects can be severe, but relatively high variance in the response of the control treatment prevented robust statistical inference from those findings. Future research on chronic multi-generational exposure is therefore advised to include a higher number of replicates to ensure statistical power of comparisons, or performing preliminary tests on assayed life-stages separately or successively. This latter recommendation is especially prudent for insect growth regulators: although these types of substances generally have little direct effect on a single life-stage (i.e., commercially produced prepupae), they may block pupation or emergence of the adult stage (Salin et al., 2003;

Singh & Johnson, 2015; Zorzetti et al., 2015), thereby hindering the reproduction that is needed for maintaining the population used for commercial production. It is argued here that the extrapolation of data from insecticide field assays to commercial rearing of BSFL (Diptera) is uncertain due to large difference in conditions. However, it is worth investigating to what extent results from studies on the control of LMW (Coleoptera) as stored grains pests may be similar to commercial rearing: a plethora of literature would be available in that case.

In Chapter 4 of this thesis, a new toxicological measure was introduced: YC_{10}, being the concentration at which exposure is expected to result in a mean reduction in yield of 10%. The YC_{10} was estimated using the 'benchmark dose' (BMD) approach, a statistical tool initially developed for human toxicological assessment (Filipsson et al., 2003). The study presented in Chapter 4 is believed to be the first in which this approach was used to model the response of insects to insecticides. The BMD approach is a risk assessment method that is used as an alternative to the No Observed Adverse Effect Level (NOAEL), which is "*the highest dose tested without evidence of an adverse effect*", and the similar parameter lowest-observed-adverse-effect-level (LOAEL) – in the absence of a tested dose without any effect (EFSA, 2009, 2017b). While the NOAEL method is dependent on specific assayed doses and experimental results, the BMD approach instead uses a fitted dose-response curve to estimate the dose at which a given effect is likely to occur using a 95% confidence interval. The BMD method allows for the calculation of confidence bounds, and the lower bound (BMDL) for a response of 5 ($BMDL_{05}$) or 10% ($BMDL_{10}$) is generally used as an acceptable benchmark for risk assessment (EFSA, 2017b; Haber et al., 2018). The BMD approach is rapidly gaining ground in ecotoxicological risk assessment (Jensen et al., 2022), and it is especially worth mentioning here a study by Yang et al. (2018) who tested the effects of several (combined) insecticides on earthworms. As the results from Chapter 4 show, the use of BMD estimations would also be an excellent tool to determine insecticide toxicity on insects reared for food and feed. The specific threshold for acceptable reductions in biomass yield in commercial insect rearing as a result of insecticide residues present in substrates would be a decision to be made at company-level. For insect rearing companies, this would necessitate striking a financial balance between higher substrate quality and lower insecticide concentrations, thereby optimizing yields on the one hand and reducing substrate costs, which may carry a risk of elevated insecticide concentrations, on the other. However, such choices may also have legal (compliance) and ethical (welfare of insects as farmed animals) implications, which are discussed further in section 5 below.

Chapter 4 also introduced a new approach for comparative toxicity analysis and assessment of joint action of multiple substances on reared insects. Firstly, the YC_{50} of a 'model' substance was estimated. Subsequently, the model substance and, in this case, also two other substances were assayed in isolation at the YC_{50} level and at 1/3 of the YC_{50} of the model substance; in a single treatment in which all three substances were combined, each at 1/3 of the model substance's YC_{50}; and finally in a treatment

combining all three substances at 1/3 YC_{50} as well as PBO. This approach provides a simple and cost-efficient way to determine the relative toxicity of different substances, as well as their combined effects when present in conjunction with one another and a synergist, and it can easily be expanded to more than three active substances. Most identified studies for insects have focused on joint action of two active substances (Robertson & Smith, 1984; Sun & Johnson, 1960a), or one active substance and a synergist such as PBO (Cetin et al., 2010; El-Guindy et al., 1983; Metcalf, 1967; Sun & Johnson, 1960b). The methodological approaches of these studies tended to be based on the theoretical framework and statistical models developed by Bliss (1939) and later expanded upon (Hewlett & Plackett, 1950, 1952; Plackett & Hewlett, 1948). This framework consists of three possible categories of interaction: independent joint action, similar joint action, and synergistic action; with the response variable being mortality. A comprehensive overview of this framework, relevant statistical models, and practical advice for experimental design are provided by Robertson et al. (2017). Research on more complex 'cocktail' effects of insecticides appears to be limited to environmental studies on aquatic animals, particularly crustaceans (Hua & Relyea, 2014; Machate et al., 2022; Nørgaard & Cedergreen, 2010; Relyea, 2009), which largely followed a similar approach as employed here, i.e., by firstly performing an exploratory assay. Validation of such initial results would require the application, or possible development, of more complex statistical models. However, an assessment of the suitability (and/or the need for adaptation) of existing models to analyse the effects of more than two insecticidal substances on a continuous variable such as biomass yield (rather than the categorical variable mortality), is beyond the scope of this thesis.

The urgency of determining toxicity effects of more complex insecticide 'cocktails' is becoming increasingly clear, both in terms of their effects on reared insects as shown in this thesis, as well as in other areas such as insects in the wild and for soil. Presented in the appendices of Chapter 4 are monitoring data collected by feed and insect rearing companies, showing residues of multiple insecticidal substances are present in individual feed materials as well as compound feeds. These data show that exposure of reared insects to multiple insecticide residues via the diet is a realistic possibility. This is confirmed by the detection of multiple pesticide residues in marketed insect products – although that could also be a result of the mixing of different batches of insect products (Kolakowski et al., 2021; Poma et al., 2017). Beyond the area of reared insects, several field studies on insects in nature reserves in the vicinity of agricultural areas have shown insect samples to be on average contaminated with over 15 pesticides (Brühl et al., 2021; Lehmann et al., 2021), and insecticide mixtures were also common in agricultural (top)soils (Geissen et al., 2021; Silva et al., 2019). Since the use of 'insecticide mixtures, mosaics or alternations / rotations' is one of the basic concepts of Integrated Pest Management (Sparks et al., 2020; Sternberg & Thomas, 2018), the likelihood of compound feeds containing a variety of many insecticide residues is estimated to be high, and increasing. This may have implications for the suitability of certain waste streams that are being considered as substrate sources for reared insects,

in particular fruit and vegetable waste (Hopkins et al., 2021; Jucker et al., 2020; Pinotti & Ottoboni, 2021). These materials tend to be comprised primarily of the outer parts (peels) that have been exposed to agricultural insecticide application, and are therefore more likely to contain different pesticides at relatively high concentrations (Nguyen et al., 2020; Taube et al., 2002).

In the studies presented in this thesis, chemical analyses of substrate, insect, and frass samples to determine concentrations of used insecticides were performed with either liquid (LC) or gas chromatography (GC) – tandem mass spectrometry (MS/MS). Generally, the LC method was employed as the standard monitoring tool, but for some substance/matrix combinations – particularly pyrethroids/ insect samples, as presented in Chapter 4 – the GC method was preferred due to higher sensitivity (Kim et al., 2022; Murcia-Morales et al., 2019). Some recent publications have detailed advances in untargeted multi-residue analytical methods for insects as a food or feed matrix specifically (De Paepe et al., 2019; Shin et al., 2020). However, since the compounds to be analysed in the insect samples were known *a priori*, seeing as how they were deliberately added to the feed, standard quality control measures were sufficient to ensure validity of the targeted analyses. This only required an extension of the accredited scope of the method, rather than elaborate analytical method development.

Insecticide residues in insect biomass

Several insect species that are reared for food and feed purposes have been observed to bioaccumulate certain food safety hazards (EFSA, 2015; Van der Fels-Klerx et al., 2018). This was most importantly shown for the heavy metals cadmium in BSFL and arsenic in YMW (Biancarosa et al., 2018; Diener et al., 2015; Van der Fels-Klerx et al., 2016), but also for some other environmental contaminants such as dioxins (Van der Fels-Klerx et al., 2020). Bioaccumulation of such hazardous compounds presents a major food safety issue for reared insects: although the concentrations may be low in the rearing substrate, elevated concentrations in the insect biomass may make the insect-derived product unsafe. In the case of pesticides, several published surveys of insect-based products that were marketed for sale as food or feed showed quantifiable levels of pesticides – presumably transferred or accumulated from the substrates or due to agricultural exposure, in the case of insects collected from the wild (Kanthawongwan et al., 2019; Kolakowski et al., 2021; Labu et al., 2022; Poma et al., 2017; Poma et al., 2022). Those findings suggest that also the presence of insecticide residues as contaminants in insect products should be a point of concern.

Table 1 shows an overview of the analysed concentrations in substrate and exposed larvae, as presented in Chapters 2 and 3 of this thesis. For most of the tested active insecticidal substances in the studies presented in this thesis, the analysed concentration in the larvae of either species was relatively low compared to the concentration in the substrate, or even below the limit of quantification (LOQ). This was especially observed for the concentrations in the LMW. However, since the MRLs of nearly all pesticides for the product type 'terrestrial invertebrate animals' are equal to

the LOQ of the analytical method, even the slightly elevated larval concentrations of the BSFL could imply non-compliance. This is problematic for those substances for which the MRLs in the substrate are substantially higher, even if low transfer rates occur, as indicated for cypermethrin and spinosad, which have a respective 40- and 100-fold difference between the MRL in wheat and the MRL in the insects. The results reported in Chapter 4 of this thesis suggest that the issue of elevated insecticide concentrations in larval biomass may be exacerbated by the presence of multiple insecticides or synergists, and higher transfer rates appear to be correlated to increased mortality. This may prove to be an advantage as it acts as a warning signal, since severely affected yields may simply prompt the commercial decision to discard the reared insect batch with the insects therefore never making it to market. Results of Chapter 5 further suggest that increased transfer rates may result from parental and/or chronic exposure. The legal implications of these findings are discussed further in section 5 below.

Table 1: Mean analysed concentrations (mg/kg) in samples of feed and larvae of black soldier fly (Hermetia illucens) and lesser mealworm (Alphitobius diaperinus) and transfer (%), calculated as larval concentration divided by substrate concentration; in comparison to maximum residue levels (MRLs) as laid down in Regulation (EC) No 396/2005 for product types wheat and terrestrial invertebrate animals (i.e. insects). Results summarized from Chapters 2 and 3 of this thesis.

Substance name	**BSFL**			**LMW**			**MRL**	
	Feed	Larvae	Transfer	Feed	Larvae	Transfer	**Wheat**	**Insects**
Chlorpyrifos	0.03	<0.005	-	0.04	<0.005	-	0.01*	0.01*
Propoxur	0.05	<0.001	-	0.04	<0.001	-	0.005*	0.01*
Imidacloprid	0.01	<0.005	-	0.12	<0.001	-	0.01*	0.01*
Spinosad	1.4	0.12	8.6%	1.58	0.001	0.1%	2.0	0.02*
Tebufenozide	0.05	<0.001	-	0.05	<0.005	-	0.01*	0.01*
Fipronil	-	-	-	0.01	n/a	-		
Pirimiphos-methyl	-	-	-	0.64	<0.001	-		
Cypermethrin	0.2	0.12	60.0%	0.38	<0.025	-	2.0	0.05*
Piperonyl butoxide	6.3	0.03	0.5%	5.1	<0.025	-	n/a	n/a

**: Indicates lower limit of analytical detection.*

An important factor in the transfer or accumulation of insecticide residues to animal tissues is the fat-solubility of the compound. This factor influences the extent to which the substance tends to concentrate in the fat or non-fat fractions (i.e., protein, carbohydrate). For reared insects, this is relevant because the fat, protein, and chitin fractions are generally separated and used for different commercial applications (Clarkson et al., 2018; Purschke et al., 2018; Saadoun et al., 2022). For instance, if insect proteins are intended to be used as feed, specific requirements on processing are laid down in Regulation (EC) No 142/2011. If an insecticidal substance tends to concentrate more in the fat or protein fraction, i.e., containing higher levels, this may have repercussions for the marketability of either product. Furthermore, the nutritional composition in terms of fat/protein ratio as well as lipid and amino acid profiles of some reared insects appear to be related to the type of rearing substrate (Franco et al., 2021; Fuso et al., 2021; Spranghers et al., 2017). It takes little speculation to anticipate

effects of insecticide residues on the composition as well, which again affects marketability. More research is accordingly needed on these subjects.

Historically, the octanol-water partition coefficient (Log P_{ow} value) is considered the 'prime indicator' for the fat solubility of a pesticide, but new evidence suggested that several other factors (also) play a role and that results from livestock metabolism studies should be considered leading in this matter (Meijer et al., 2020). Nonetheless, both Houbraken et al. (2016) and Dreassi et al. (2020) found that pesticides with higher Log P_{ow} values tended to accumulate in and be retained to a higher degree in YMW, as compared to pesticides with lower Log P_{ow} values, which suggests that this factor does play a major role in the uptake of pesticides by reared insects. In Regulation (EC) No 396/2005, pesticides which are considered fat soluble are marked with '(F'), following the pesticide designation. The majority of substances that were tested in the research presented in this thesis are marked as fat soluble: all pyrethroids (cypermethrin, deltamethrin, permethrin), pirimiphos-methyl, chlorpyrifos and chlorpyrifos-methyl, spinosad, tebufenozide, and fipronil. Lacking this designation, and therefore presumed to be not or much less fat-soluble, are malathion, propoxur, and imidacloprid. The data presented in this thesis largely confirm that the fat soluble substances were transferred to a higher degree to the BSFL biomass than those lacking the (F) designation. Transfer for LMW was low overall, despite comparatively similar fat contents described in literature (Janssen et al., 2017), which suggests that a different factor than fat solubility of tested compounds determined transfer rate for LMW.

In the case of many organic contaminants that may be present in the substrate of reared insects, the parent compound may be broken down into certain metabolic products. These may be more or less toxic for the insect, or for the subsequent consumer, than the parent compound. For instance, organothiophosphates require oxidative bio-activation into the toxic oxon form for their insecticidal effect (Vale, 2015; Van Dyk & Pletschke, 2011). Conversely, in the case of, for instance aflatoxin B_1 (AFB_1), the parent compound is to some extent metabolized by YMW and LMW into the M_1 metabolite and by BSFL into AFP_1 and aflatoxicol, which are all still toxic – although the insects themselves did not appear to be adversely affected (Bosch et al., 2017; Camenzuli et al., 2018; Meijer et al., 2019). In the research presented in this thesis, formation of metabolic breakdown products from tested insecticides were out of scope. I recommend that this is part of future research: firstly, because knowledge on the metabolism of insecticides by reared insects could provide valuable insight into (cross-)resistance mechanisms, and secondly because the presence of potentially toxic metabolites in insect biomass could present a food safety issue. I would suggest that the legal definitions which may contain specific mention of metabolites and isomers of tested insecticides are used as a starting point for such follow-up research. For some of the tested substances, the pesticide residue definition already includes multiple compounds, this being the case for spinosad (sum of spinosyn A and spinosyn D), fipronil (sum fipronil + sulfone metabolite (MB46136) expressed as fipronil), malathion (sum of malathion and malaoxon expressed as malathion), permethrin (sum of

isomers), deltamethrin (*cis*-deltamethrin), and cypermethrin (cypermethrin including other mixtures of constituent isomers (sum of isomers)). Cypermethrin formulations are composed of eight *cis*- and *trans*-stereoisomers, which are more or less bioactive compared to one another. Depending on the formulation, it is classified as alpha-, beta-, or zeta-cypermethrin. For instance in case more than 90% of the mixture consists of the more active *cis*-isomers, it is classified as alpha-cypermethrin. At this time, only cypermethrin formulations with a maximum *cis*-isomer content between 40 and 60% are permitted (EFSA, 2023). In the studies presented in this thesis, generally only the parent compound was added to the substrates, with the exception of spinosad which was used as a mixture of spinosyn A and D present at 76.9 and 23.1 %, respectively. In some other cases such as chlorpyrifos-methyl, the primary breakdown product and potentially toxic metabolite desmethyl chlorpyrifos-methyl is not explicitly mentioned in the pesticide definition, but the substance is rather marked with an 'R', denoting that the residue definition differs for certain specified product groups. Inclusion of such additionally specified metabolites is required in chemical analysis for monitoring purposes (EFSA, 2017a). Methodologically, high resolution mass spectrometry (HRMS) coupled with metabolomic analysis could provide key data for targeted and untargeted determination of metabolic breakdown products of insecticide residues by reared insects (De Paepe et al., 2019).

Legal implications

Legally, insects reared for food and feed are considered 'farmed animals' in the context of Regulation (EC) No 1069/2009 (Article 3.6(a)): a designation that places specific requirements on the substrates (in particular those of animal origin) on which the insects may be reared, and the types of animals to which such insects may be fed. However, not all EU agricultural rules apply to insects to the same extent as they do to many other farmed animals: insects, being invertebrate animals, are explicitly exempt from Directive 98/58/EC concerning the protection of animals kept for farming purposes (Article 1.2(d)). It must be noted that, in contrast to Regulations, Directives do not have direct effect, but must instead be transposed by each EU Member State into its own national legal system. There has been a recent increase in research on the extent to which (reared) insects are sentient: the current consensus appears to be that although there is no definitive answer to that question yet, application of the 'precautionary principle' is advised and steps should be taken to minimize suffering (Barrett et al., 2023; Delvendahl et al., 2022; Kortsmit et al., 2023; Parodi et al., 2022; RDA, 2018; Van Huis, 2019; Voulgari-Kokota et al., 2023). As such, substrates containing insecticide residues that adversely affect survival and growth of reared insects are unsuitable from an ethical point of view. As such, certain countries may have additional rules on animal welfare that also cover reared insects. For instance, in the Netherlands, animal welfare safeguards are used as a criterion in the assessment of an application for a dispensation to rear BSFL (see, e.g., (RVO, 2021)). Nonetheless, it must be concluded that EU-level animal welfare rules for insects are largely absent. The International Platform of Insects for Food and Feed (IPIFF) has published a memo on

animal welfare for reared insects (IPIFF, 2022a), but adherence to this guide is of course optional.

However, it is argued here that a different indirect legal route does provide certain ethical protections for reared insects. Since insects reared for food are considered 'food-producing animals', they are subject to all provisions of food law. The feed on which they are reared may therefore not be unsafe (Articles 3.1 and 15 of Regulation (EC) No 178/2002). Feed is unsafe if, taking into account the intended use: 1) has an 'adverse effect on human or animal health', or; 2) 'make the food derived from food-producing animals unsafe for human consumption' (Article 15.2 of Regulation (EC) No 178/2002). These feed safety requirements also apply to insects reared for feed purposes as 'non-food producing animals' (Article 3.2(d) and 4.1(b) of Regulation (EC) No 767/2009). These two Regulations (178/2002 and 767/2009) read in conjunction with one another are interpreted to mean that the feed for both food- and non-food producing insects may thus not be 'unsafe'. In the following two sub-sections, the two requirements for safe feed are discussed in relation to the observed adverse effects on the performance and welfare of reared insects.

Safety of insecticide residues in substrates for insects as farmed animals

The first legal requirement for safe feed is that it does not cause adverse effects on animal health. The experimental results presented in this thesis provide evidence that this requirement is not met when reared insects are exposed to residues of certain insecticides – even at concentrations below the applicable legal limit – since survival and yields have been shown to be adversely affected. The monitoring data presented in the Supplementary Materials of Chapter 4 demonstrate that insecticide residues are present in substrates at such levels. Although any feed containing such levels of insecticides may be compliant with the MRL, it would still normatively be unsafe – if intended for an insect species that is adversely affected by such concentrations. Therefore, such contaminated feed does not comply with food law and may therefore not be fed to such insects, or even placed on the market if specifically intended for these insect species.

This posited non-compliance with food law in case of insecticide residues having a likely adverse effect on insects reared for food or feed begs the question in what manner, if at all, this should be remediated. In terms of legal recourse, lower MRLs specifically for feed materials intended for reared insects could be considered. For instance, by moving the MRL for cypermethrin in wheat from 2.0 mg/kg to the estimated CED_{10} for BSFL of 0.4 mg/kg, as discussed in Chapter 4. Although Regulation (EC) No 396/2005 is the established framework Regulation for pesticide MRLs, these MRLs apply only to commodity-substance combinations and this Regulation does not differentiate between materials intended as food or feed – let alone between different intended farmed animals. MRLs laid down in Directive 2002/32/EC do allow for this differentiation between different categories of animals, and this Directive therefore seems to be a more

suitable instrument for insect substrate-specific limits on insecticide residues. At this time, however, there are too many scientific uncertainties for such limits to be implemented in law. Of the large number of insecticides that are permitted to be used in the EU, for only relatively few have the effects been tested on reared insects, in the studies presented in this thesis, and a few similar studies present in scientific literature. Of course these results would also need to be replicated, including by assaying different commercial populations. Furthermore, the potential for cocktail and sub-lethal effects, as discussed above, introduces complexities that hinder any short-term implementation of definitive legal limits.

An alternative mechanism to legal limits could be the development of guidance levels that can be implemented via contractual agreements between insect rearing organizations and their substrate suppliers. If such guidance is disseminated widely – for instance via the Guide for Good Practice developed by IPIFF and endorsed by the European Commission's Standing Committees on Plants, Animals, Food and Feed ('Biological safety of the food chain' and 'Animal nutrition') (IPIFF, 2022b) – then the general objective of harmonization of rules to ensure free movement of goods could still be met. Enforcement by competent authorities in case of egregious non-compliance - e.g., wilful exposure to insecticide residues that would cause excessive mortality – could of course still be done based on the aforementioned general principle of safety (Article 15.2 of Regulation (EC) No 178/2002). Finally, the findings presented in this thesis could provide justification for an expedited implementation of organic certification for reared insect products (IPIFF, 2021), which should require the use of organically sourced substrates – although the permitted use of spinosad in organic production, as discussed in Chapter 3 and 5, would then need revaluation.

Safety of insecticide residues in insect biomass as feed for other farmed animals or as food

When reared insects are used as feed, they generally comprise one of multiple ingredients in compound feeds for production animals such as pigs, poultry and aquaculture animals. For instance in the case of nutrition for aquaculture animals such as salmon, reared insect meal can (partly) replace fishmeal wild-caught fish (Gasco et al., 2020; Henry et al., 2015; Schiavone et al., 2017). Therefore, even elevated insecticide concentrations in the insect biomass could be offset by the dilution with other ingredients when mixed. However, Article 19 of Regulation (EC) No 396/2005 explicitly prohibits the mixing or processing of non-compliant products for purposes of dilution. It is unclear what the reason is for the setting of most insecticide MRLs in the product type 'insects' (1060000) at the default level: inspired by legitimate safety concerns, or a lack of data. Of course, any decision to increase the current MRLs for insects should consider the possible proportion of insect-based products in compound feeds intended for other production animals, and the potential associated pesticide intake of those animals. For instance, care should especially be taken in case of pyrethroids such as cypermethrin, which are highly toxic to many aquatic animals (Farag et al., 2021),

which justifies a precautionary approach. A full risk assessment by EFSA is thus recommended.

In terms of the effects of insecticide residues on reared insects as presented in this thesis, the second requirement of Article 15.2 of Regulation (EC) No 178/2002 relates to the potential bioaccumulation or transfer of insecticide residues from substrate to insect biomass. This provision states, in summary, that the final concentration in the insects may not be so high so as to make it 'unsafe' for human consumption. This requirement is implemented via the specific insecticide MRLs laid down in Regulation (EC) No 396/2005 for the product category of terrestrial invertebrate animals, which are mostly set at the substance-specific default of approximately 0.01 mg/kg, as discussed in Section 4 above. Exceedance of these MRLs would thus imply non-compliance of the particular insect-derived food product.

The MRLs defined in Regulation (EC) No 396/2005 do not only apply to materials intended as animal feed, but (arguably) primarily to food for human consumption. According to preamble 5, MRLs are set at the "*lowest achievable level consistent with good agricultural practice*" [GAP], but "*with a view to protecting vulnerable groups such as children and the unborn*". The principle is implemented via a mandated consideration of the risks of exceedance of the acceptable daily intake (ADI) or acute reference dose (ARfD) in the assessment of MRLs by the European Food Safety Authority (EFSA) (Article 10 of Regulation (EC) No 396/2005). As far as I am aware, this assessment has not been performed for insecticide MRLs in insect products. Given the likelihood of non-compliance of insects that have been exposed to dietary insecticide residues, as presented in this thesis, it is recommended that any potential risks – or their absence – are to be assessed by EFSA, and that MRLs are revised accordingly.

Recommendations and future outlook

At the time of writing of this final Chapter of my thesis, July 2023, the EU commercial insect rearing industry is in a state of flux. Some companies have recently switched to rearing of different species, moved their production overseas, defaulted, or closed for other reasons. At the same time, new start-ups have sprung up to fill certain niches within the industry. The volume of scientific literature on the subject of insects for feed and food has grown almost exponentially in the 10 years that have passed since the FAO publication by Van Huis et al. (2013) (Boukid et al., 2023; Van Huis, 2023). Still, the predicted large-scale production and consumption of reared insect products in Europe has not yet come to pass, in part due to a variety of (legal) barriers that have remained in place or lifted only partially (Niyonsaba et al., 2023; Veldkamp et al., 2022). Many studies have focused on determining the performance of reared insects on a variety of substrates to optimize yields (e.g. looking into particle size and moisture content of substrates), and/or pave the way for new types of substrates that are at this time not yet allowed (Gligorescu et al., 2020; Holmes et al., 2013; Ribeiro et al., 2022; Siddiqui et al., 2022; Veldkamp et al., 2021). In contrast, the studies presented in this thesis, as well as some performed by other authors, have shown that the presence of

insecticide residues in insect substrates can have significantly adverse effects on those same yields, thereby possibly negating the yield gains from optimized substrate compositions. Furthermore, it has been shown that these effects can be problematic for reasons of food and feed safety, and of course for the welfare of the reared insects.

More research is needed on a variety of subjects related to the effects of insecticide residues on reared insects. Firstly, the scope of the research presented in this thesis was limited to two insect species and twelve insecticidal active substances, against eight species currently allowed to be processed as feed and over 450 permitted pesticides (Heinrich-Böll-Stiftung, 2022). Expansion of this scope is therefore needed, since the results presented here suggest that the effects of different substances – even those with the same MoA – are not comparable, nor are the responses of different species to the same type of exposure. Furthermore, the presence of insecticide cocktails in compound feeds and their potential for joint or synergistic action is especially worrisome and requires urgent elucidation. The preliminary findings on sub-lethal effects presented in Chapter 4 (for neonate BSFL) and Chapter 5 (for the entire LWM lifecycle), suggest that different life-stages can be more or less sensitive than the prepupae that are generally used for production. Again, the scope was limited, but these initial findings provide sufficient and compelling justification for follow-up research. Finally, the potential for substances to become concentrated in certain fractions, possibly depending on certain physico-chemical properties, is a point that requires more investigation.

The need for more research does not preclude both policy and industrial stakeholders from taking short-term actions to reduce the observed effects of insecticide residues on reared insects. Firstly, the research presented in this thesis provides ample evidence that insecticide concentrations at or below the MRL could adversely affect yields and insect welfare. Insects are classified as farmed animals and should therefore be granted similar protection as other farmed animals: current insecticide MRLs are not fit for purpose in that regard. In the absence of acceptable legal limits for substrates, insect rearing organizations are advised to include lower acceptable limits in contractual agreements with their substrate suppliers. An alternative to company-specific contractual limits would be to instead adopt guidance levels in the industry-wide Guide for Good Practice that has been developed by IPIFF. Of course, this does not absolve policymakers from considering lower MRLs specifically for insect substrates. In the opinion of this author, Directive 2002/32/EC would be the most suitable instrument for such limits. In terms of food and feed safety, there is a reasonable likelihood of insecticide transfer from substrate to insect biomass resulting in exceedance of MRLs in insect products – since those MRLs are largely set at the substance-specific default. In addition to revision of MRLs in insect substrates, the current default limits for virtually all pesticides in insect-based products should therefore also be reassessed. Depending on the level of risk, these MRLs could be increased from the current default to more attainable limits. Finally, I would argue that the best way to mitigate the effects of insecticide residues on reared insects is simply by way of comprehensive reductions in agricultural use, as proposed in the EU Green Deal: not only to safeguard biodiversity,

ecosystems, health and the environment, but thus also to protect insects reared for food and feed.

In conclusion, the experimental research presented in this thesis has shown that the presence of insecticide residues in the substrate on which edible insects are reared can have adverse effects on these farmed animals. These adverse effects are primarily of a sub-lethal nature, with reduced total biomass (yield) as the main indicator – although significant increases in mortality have also been observed. Exposure to multiple substances ('cocktails') can augment these effects considerably and the potential for additional sub-lethal effects on reproductive capacity resulting from chronic multi-generational exposure cannot be excluded. Finally, exposure to insecticide residues present at the maximum allowed legal limit in feed can be transferred to the insect biomass to an extent that it makes the insect-based product uncompliant with comparatively lower insect-specific legal limits. More research is needed and urgent remedial action by policymakers and industry is advised.

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Summary

Insects reared specifically for purposes of food and animal feed are considered to be a promising new alternative that can contribute to the increasing global demand for sustainable proteins. Due to the limited history of use of insects as food and feed in the European Union (EU), a precautionary approach is employed and certain safety assurances must be met before insect-based products can legally be marketed for feed or food in Europe. Many safety aspects related to use of insects as food or feed are at this time still uncertain. One particular category of hazards that has received comparatively limited attention until now are insecticides, which may be present in feed materials as residues from agricultural use. Insecticide residues in the substrate on which insects are reared may affect their growth and survival, thereby impacting the health of insects and the profitability of the insect industry. In addition, there is a potential risk of bioaccumulation of contaminants in the insects from the substrate on which they feed. At this time in the EU, a total of eight insect species are permitted to be used as 'processed animal protein' (PAP) in feed for aquaculture animals, and pigs and poultry. Black soldier fly larvae (BSFL, *Hermetia illucens* (L.); Diptera: Stratiomyidae) and lesser mealworm (LMW, *Alphitobius diaperinus* (Panzer); Coleoptera: Tenebrionidae) were the focus of the research performed for this thesis.

Different insecticidal substances may interact with one another: additively or synergistically, or antagonistically. Secondly, other stages in the insect lifecycle than the larval stage may be more or less affected by exposure to insecticides, or performance may be affected by exposure in a previous life stage. The primary objective of this thesis was to assess the effects of insecticide residues present in the feed substrate on BSFL and LMW performance and bioaccumulation. This primary objective was split into two sub-objectives: firstly, to assay the effects of selected insecticides on these two insect species, primarily in terms of survival and growth that both determine biomass yield. The second sub-objective was to determine transfer or accumulation of tested insecticidal substances from the substrate to the insect biomass, to be used for food or feed purposes.

In the first study of this project (Chapter 2), the effects of exposure of BSFL via the feed substrate to six insecticidal substances (chlorpyrifos, propoxur, cypermethrin, imidacloprid, spinosad, tebufenozide), each with different modes of action were tested in two sequential experiments. Cypermethrin was also tested with the synergist piperonyl butoxide (PBO). Standard BSFL substrate was spiked to the respective maximum residue level (MRL) of each insecticide allowed by the European Union to occur in feed, and BSFL were reared on these substrates. Insecticide concentrations in the spiked substrate and larvae were determined by LC-MS/MS. Depending on the observed effects in the first experiment, spiked concentrations tested in the second experiment were increased or reduced. At the concentrations applied (1 and 10 times MRL), three of the six tested substances (chlorpyrifos, propoxur, tebufenozide) did not affect the survival or growth of BSFL, compared to the control (non-spiked) treatments. At MRL, imidacloprid stimulated the growth of BSFL compared to the controls. Spinosad and cypermethrin at the MRL level negatively affected survival and growth. The effects

of cypermethrin appeared to be augmented by addition of PBO. A mean bio-accumulation factor of ≤0.01 was found in both experiments for all substances, except for cypermethrin, which was comparatively high, but still below 1 (0.79 at 0.1 mg/kg).

The second experimental study (Chapter 3) focused on the determination of the effects of insecticide residues on LMW, again in terms of survival and growth as well as substance transfer. Tested substances were the same as in the first study on BSFL, in addition to fipronil and pirimiphos methyl. Concentrations in the larvae were largely below the limit of quantification, meaning that bioaccumulation did not occur and larval biomass was compliant. Significant reductions in total yield were observed for spinosad (present in the substrate at 1.6 mg/kg) and imidacloprid (0.12 mg/kg). Spinosad is one of few insecticides that is permitted to be used in organic agriculture, which raised questions over the safety of organic produce for insect rearing.

The third study (Chapter 4) aimed to investigate the effects of six different insecticides belonging to the pyrethroid and organophosphate classes on the performance of this insect species. 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 for pirimiphos-methyl (4.8 mg/kg) were determined. The addition of PBO to cypermethrin enhanced its relative potency with a factor 2.6. At a cypermethrin to PBO ratio of 1:20, the CED10 was 0.2 mg/kg. 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. Negative effects were also observed for exposure to multiple substances at lower concentrations, suggesting additive and synergistic effects. Of the tested substances, deltamethrin was most toxic, causing 94% yield reduction even at 0.5 mg/kg. Analytical results suggest 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.

In the fourth and final experimental study (Chapter 5), two generations of *A. diaperinus* were chronically exposed to spinosad (2.0 and 0.2 mg/kg) and imidacloprid (0.1 and 0.01 mg/kg) in the substrate. The aim was to determine sub-lethal effects on performance measures (total biomass (yield), mean individual weight, number of alive individuals) of larvae, pupae, and adult beetles, as well as pupation and eclosion. Selection of these substances and concentrations was based on the findings of the first study on LMW discussed above. Exposure to spinosad at 2.0 mg/kg resulted in significant adverse effects on most performance measures of larvae, of both generations. Imidacloprid caused a reduction in yield and mean individual weight of the larvae of the parent generation, as compared to the control at 0.1 mg/kg, while an increase in those measures was observed at 0.01 mg/kg. Conversely, significant

adverse effects on adult beetles only of the offspring generation were only observed for imidacloprid at 0.1 mg/kg, and no significant effects of this insecticide on pupation and eclosion were observed. The concentrations of tested substances in larval samples were negligible for both generations, however, transfer from substrate to larval biomass was higher in the offspring generation relative to the parent generation.

In conclusion, the experimental research presented in this thesis has shown that the presence of insecticide residues in the substrate on which edible insects are reared can have adverse effects on these farmed animals. These adverse effects are primarily of a sub-lethal nature, with reduced total biomass (yield) as the main indicator – although significant increases in mortality have also been observed. Exposure to multiple substances ('cocktails') can augment these effects considerably and the potential for additional sub-lethal effects on reproductive capacity resulting from chronic multi-generational exposure cannot be excluded. Finally, exposure to insecticide residues present at the maximum allowed legal limit in feed can be transferred to the insect biomass to an extent that it makes the insect-based product uncompliant with comparatively lower insect-specific legal limits. More research is needed and urgent remedial action by policymakers and industry is advised.

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About the author

Curriculum Vitae

Nathan Pepijn Meijer was born on Sunday the 24th of April 1988 in Leeuwarden, The Netherlands. After moving to his new home in Groningen, he finished his secondary education (VWO) at the Augustinus College. He finished his Bachelor of Commerce degree in Food & Business in 2012 at the Hanzehogeschool Groningen, during which he did internships in Kuala Lumpur (Malaysia) and London (United Kingdom). In 2014, he started the MSc. programme Food Safety Law at Wageningen University. He did his internship at the Authenticity & Nutrients department of Wageningen Food Safety Research (WFSR, formerly RIKILT). After successfully obtaining his MSc. Degree in 2016, he started working as a researcher at the WFSR Agrochains group where he presently still works. In this capacity, he has coordinated or assisted in the management of large national and international scientific projects, such as the Horizon 2020 projects SUSINCHAIN and HOLiFOOD. In 2019, he started his PhD research under the supervision of Prof. dr. ir. J.J.A. van Loon and Prof. dr. ir. H.J. van der Fels-Klerx, at the Wageningen University Laboratory of Entomology. In this research, he has focused on the effects of insecticide residues on insect species reared for food and feed. In addition, he has (co-)authored multiple peer-reviewed scientific papers on a variety of subjects related to food safety and legislation. His passions include photography of many different subjects, including the insects and people that have been photographed for this thesis.

List of publications

In peer reviewed journals

Meijer, N., Zoet, L., de Rijk, T., Zomer, P., Rijkers, D., Van der Fels-Klerx, H.J., Van Loon, J.J.A. (2023). Effects of pyrethroid and organophosphate insecticides on reared black soldier fly larvae *Hermetia illucens*. *Insect Science.* https://doi.org/10.1111/1744-7917.13269 (**Chapter 4 in this thesis**)

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Submitted

Van der Borg, G., Hosseini, A., Van Raamsdonk, L.W.D., Zheng, R., Schmitt, E., Hedemann, B., Ruis, S., Van Bemmel, G., Dam, N., Meijer, N. (submitted) Transfer of microplastics from packaging materials to black soldier fly larvae (*Hermetia illucens*) used for food and feed.

Meijer, N., Bosch, M.W., de Rijk, T., Zomer, P., Van der Fels-Klerx, H.J., Van Loon, J.J.A. (submitted). Lethal and sublethal effects of chronic exposure to insecticide residues on reared *Alphitobius diaperinus*. (**Chapter 5 in this thesis**)

Mulder, P.; Müller-Maatsch, J.; Meijer, N.; Bosch, M.; Zoet, L.; Van der Fels-Klerx, H.J. (submitted). Effects of dietary exposure to plant toxins on bioaccumulation, survival, and growth of black soldier fly *Hermetia illucens* larvae and lesser mealworm *Alphitobius diaperinus*.

Hoek–Van den Hil, E.F., Meijer, N., Van Rozen, K., Elissen, H., Van Wikselaar, P.G., Brust, H., Te Loeke, N.A.J.M., de Rijk, T., Tienstra, M., Van de Schans, M.G.M., Wanrooij, J., Van der Weide, R., Veldkamp, T., Van der Fels-Klerx, H.J. (submitted). Safety of black soldier fly (*Hermetia illucens*) larvae reared on waste streams of animal and vegetal origin and manure.

Niermans, K.; Meijer, N.; Hoek, E.; Nijssen, R.; Zoet, L.; Boers, E; Van Loon, J.J.A.; Van der Fels-Klerx, H.J. (submitted). The metabolic fate and biological effects of isotopically labelled aflatoxin B1, fumonisin B1, ochratoxin A and zearalenone in reared *Hermetia illucens* and *Musca domestica* larvae.

Training and Education statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review/project proposal (9 ECTS)
- Health risks and EU regulatory framework
- Chemical safety of insects for food and feed use

Post-graduate courses (4 ECTS)
- Summer school insects as food and feed from producing to consuming; Wageningen Academy (2019)
- Basic statistics; PE&RC (2021)

Invited review of (unpublished) journal manuscript (2 ECTS)
- Journal of Insects as Feed and Food: the house cricket (*Acheta domestica*) as a novel food: a risk profile (2018)
- Food Control: a review on risks assessments of *Tenebrionidae* and *Gryllidae* in relation to a first machines and plants development (2019)

Competence strengthening/skills courses (2 ECTS)
- Essentials course in project management; WUR (2017)
- IPMA D certification course; WUR (2018)

Scientific integrity/ethics in science activities (0.7 ECTS)
- Scientific integrity session; WUR (2020)
- Scientific integrity; WUR (2022)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)
- PE&RC First years weekend (2019)
- PE&RC Last years weekend (2022)

Discussion groups/local seminars or scientific meetings (7 ECTS)
- Expertmeeting cirkelwaarde demonstratieproject larven kweek soldatenvlieg op groenten en fruitafval van huishoudens, horeca- en cateringafval (2018)
- Chain group presentations; Rikilt (2018-2019)
- Insect meeting; WUR (2018-2020)
- Mini-symposium insecten; HAS Hogeschool Den Bosch (2019)
- Visie op de insectensector in Nederland (2019)
- H2020 Project Susinchain's consortium meeting (2019-2021)
- Symposium on biological pest control (2021)

International symposia, workshops and conferences (7.7 ECTS)
- Insecta conference; oral presentation; Berlin, Germany (2017)
- Insects as feed ingredient in poultry farming; oral presentation; Legnaro, Italy (2018)
- Feed conference; oral presentation; Bergen, Norway (2018)
- Insecta conference; oral presentation; Magdeburg, Germany (2021)
- EAAP Meeting; Porto, Portugal (2022)

Lecturing/supervision of practicals/tutorials (0.9 ECTS)
- Insects for food and feed (2020, 2021, 2022)

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