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RESEARCH ARTICLE

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

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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 sublethal 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 sublethal effects on *A. diaperinus*, for which method development for more cost-efficient designs is required.

Keywords

pesticides – reproduction – chronic exposure – mealworms

1 Introduction

Reared insects are increasingly seen as a suitable alternative protein source for food and feed (Hawkey *et al.*, 2021; Sogari *et al.*, 2019; Van Huis, 2020, 2021a). 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

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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 spinosad and imidacloprid 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 (Hertlein *et al.*, 2011; Huang and Subramanyam, 2007; Nayak *et al.*, 2005), and *A. diaperinus* in particular (Lambkin and Furlong, 2014; Lambkin and Rice, 2007; Mustač *et al.*, 2013; Singh and Johnson, 2015; Tomberlin *et al.*, 2014; 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 spinosad and imidacloprid. 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.

2 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 trip-

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	Spinosad	94.0%	168316-95-8	0.2
3	Imidacloprid	98.55%	138261-41-3	0.10
4	Imidacloprid	98.55%	138261-41-3	0.01
5	Control	n/a	n/a	n/a

licate, 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.

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. Since the feed was of organic quality (producer certified by Dutch certifying body SKAL, Regulation (EU) 2018/848), the absence of synthetic insecticide residues other than spinosad and imidacloprid was not further verified. 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 equalled 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 Tabletop 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 analysed to determine potential degradation of the active insecticidal substance in the feed throughout the experiment.

Experimental procedures

At this time, insects, being invertebrate animals, are exempt from Directive 98/58/EC concerning the protection of animals kept for farming purposes as well as legislation on animals used for scientific purposes (Directive 2010/63/EU). Nonetheless, steps have been taken to reduce insect suffering in the experimental design; e.g. by freezing the larvae as a killing step (van Huis, 2021b).

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 3,000 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

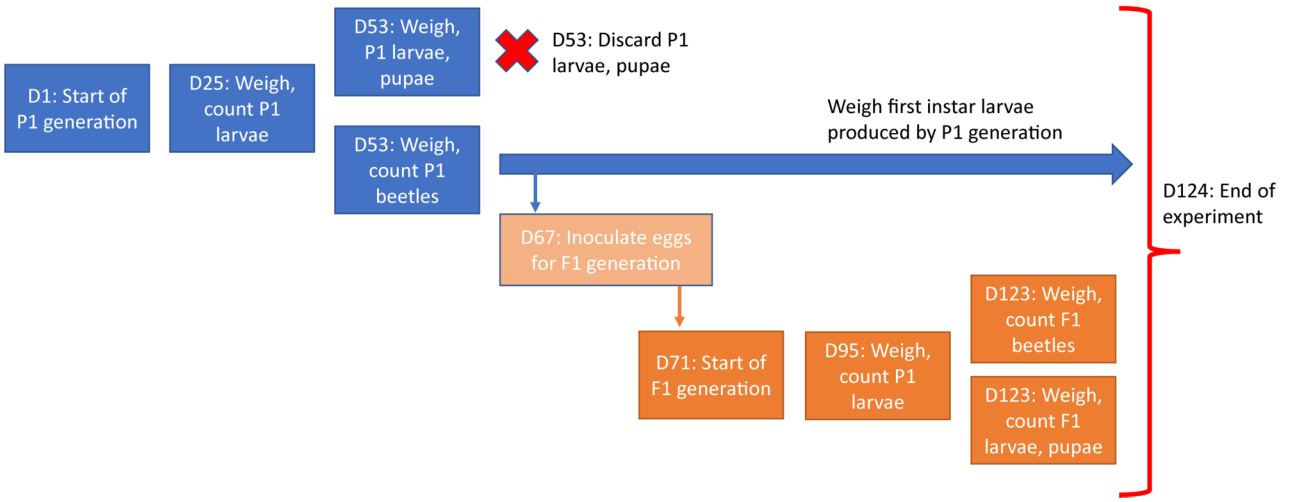

FIGURE 1 Schematic depiction of bioassay. Procedures performed on the parent (P1) generation are shown in blue; for the first offspring generation (F1) in orange. D represents the number of days since the start of the experiment.

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

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.

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 3,600 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 S1. The method was capable of detecting imidacloprid, spinosyn A and spinosyn D (together "spinosad") at a level of 0.001 mg/kg each. The lowest validation level was 0.010 mg/kg each.

Of the three batches of feed prepared for this experiment, the second batch (randomly determined) was used to analyse 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 analysed 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 analysed 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).

3 Results

Quality control

Analysed concentrations of spiked substances in the feed are shown in Supplementary Table S2. Overall, analysed 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 and that deviations were due to a minor human error. 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 analysed.

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 Supplementary 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) ($P \leq 0.05$).

Insect performance – 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 Supplementary 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 ($P \leq 0.05$).

Insect performance – 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

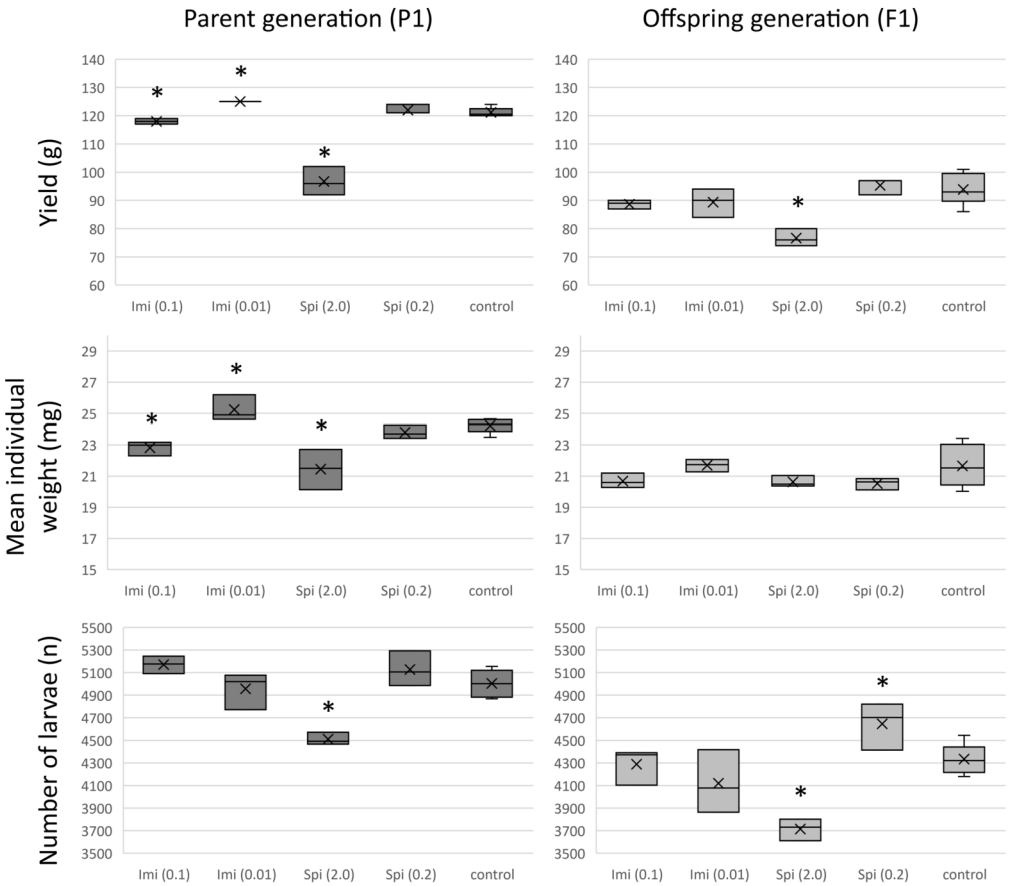

FIGURE 2 Yield (g), mean individual weight (mg), and number of individual larvae (n) of the first (P1, left) and second generation (F1, right). Mean and standard deviation of n = 3 replicates for treatments and n = 6 for the control. Asterisks (*) denote significant differences between a treatment and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

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.

Insect performance – 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.

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, concentrations in the larval biomass was <0.01 mg/kg, resulting in transfer of tested substances being below 10%.

4 Discussion

Results of this study on direct lethal and sublethal 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 decemlin-*

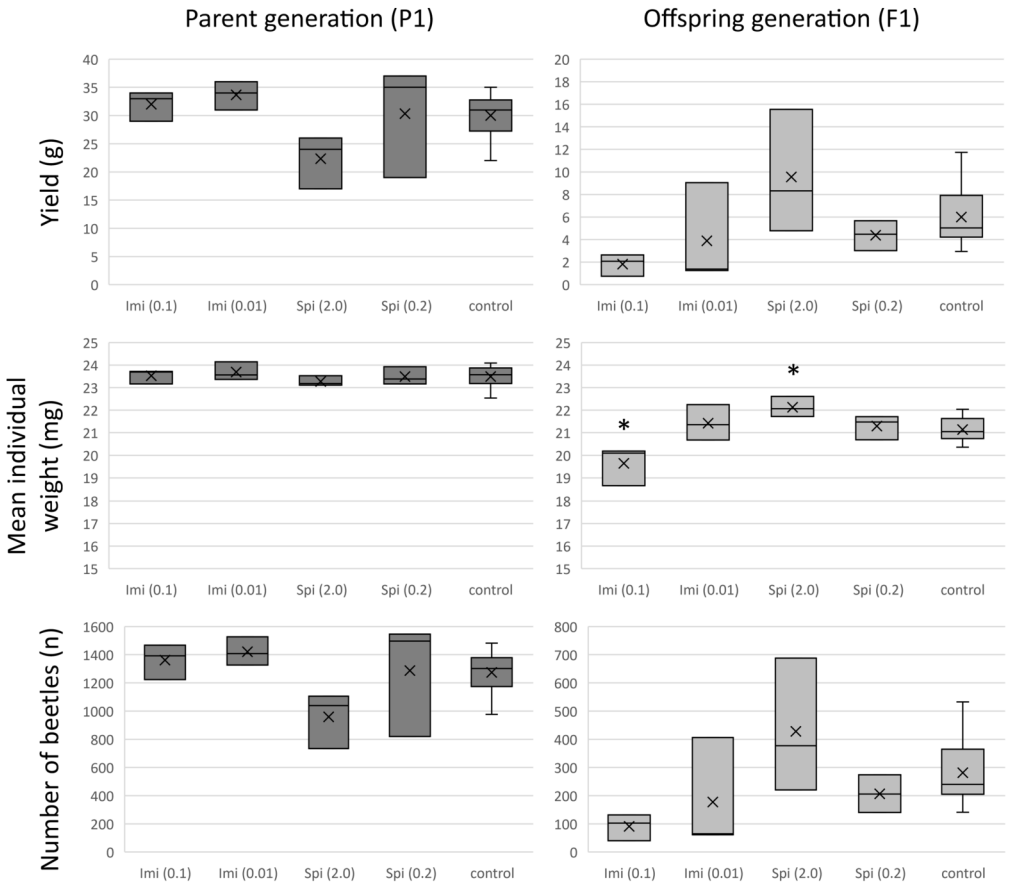

FIGURE 3 Yield (g), mean individual weight (mg), and number of beetles alive (n) of the first (P1, left) and second generation (F1, right). Mean and standard deviation of n = 3 replicates for treatments and n = 6 for the control. Asterisks (*) denote significant differences between a treatment and the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

TABLE 3 Analysed concentrations (mg/kg) of spiked substances spinosad and imidacloprid 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)			
Spinosad (2.0)	1.9	0.006	0.3%
Spinosad (0.2)	0.14	0.000	0.1%
Imidacloprid (0.10)	0.094	0.001	1.5%
Imidacloprid (0.01)	0.020	0.001	3.1%
Generation 2 (F1)			
Spinosad (2.0)	2.0	0.010	0.5%
Spinosad (0.2)	0.12	0.001	0.6%
Imidacloprid (0.10)	0.082	0.003	3.2%
Imidacloprid (0.01)	0.011	0.001	6.9%

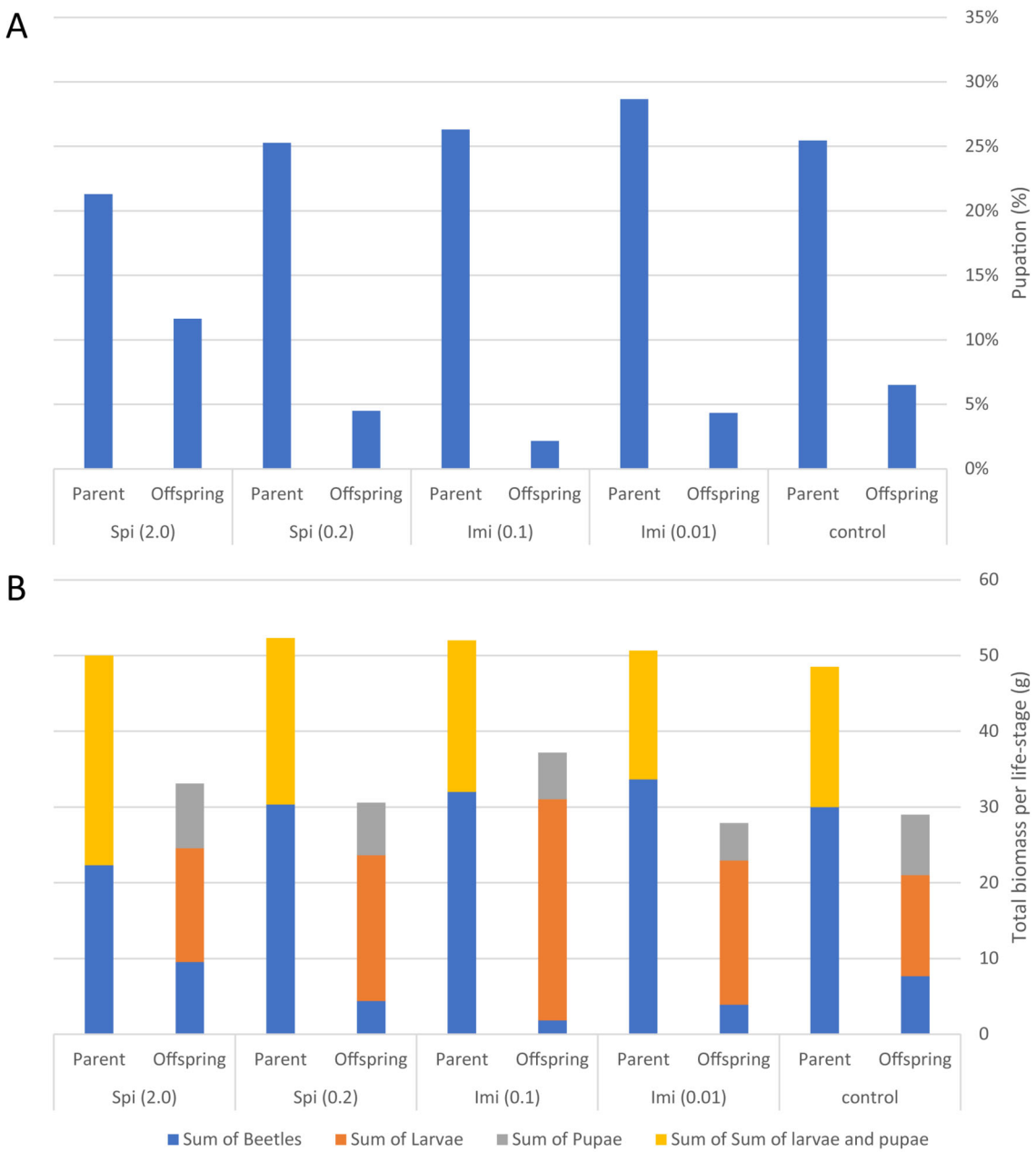

FIGURE 4 Pupation for parent (P1) and offspring (F1) generations: the number of beetles on day 48 after eclosion as a percentage of the number of larvae on day 25. (B) Total biomass (g) per life-stage: beetles, larvae, and pupae for P1; beetles and sum of larvae and pupae for F1.

eata (Say); Coleoptera: Chrysomelidae) (Bažok *et al.*, 2008; Igrc Barčić *et al.*, 2006). 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.

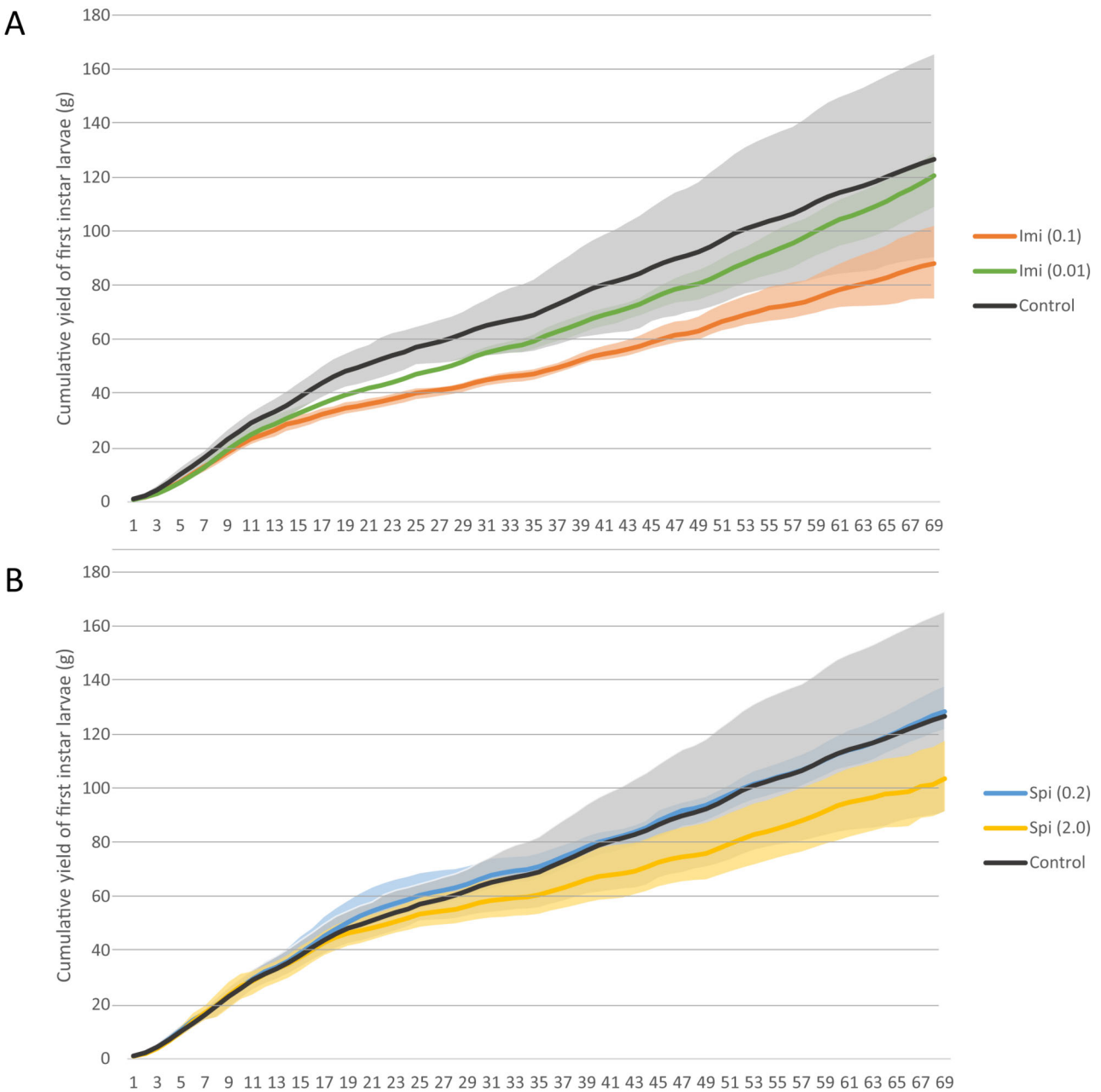

FIGURE 5 Stacked line chart of cumulative yield (g) of first instar larvae (after eclosion) per day per 100 g of parent beetles producing the F1 generation. (A) shows data for imidacloprid at 0.1 and 0.01 mg/kg; (B) shows data for spinosad at 0.2 and 2.0 mg/kg; both figures show the control data. Arithmetic 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.

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 (Mota-Sanchez *et al.*, 2006; Olson *et al.*, 2000; Zhao *et al.*, 2000). 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. Alternatively, different substrate compositions that have been optimized for each of the assayed lifecycles could be used, although the logistical complexity of this could be prohibitive.

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 spinosad and imidacloprid 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,b). As such, we recommend that alternative bioassay methods are developed to determine their use in testing for sublethal 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 sublethal effects have employed cameras and software to assess the (larval) motility response to insecticidal exposure (Denecke *et al.*, 2015; Tooming *et al.*, 2014). 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 sublethal effects of insecticides, particularly neonicotinoids such as imidacloprid, on honey bees in relation to Colony Collapse Disorder (Chambers *et al.*, 2019; De Smet *et al.*, 2017; Wu-Smart and Spivak, 2016), 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.

5 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 spinosad and imidacloprid. 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 (as an indirect effect) 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 sublethal effects to reared insect populations of *A. diaperinus*, as well as other species that are being reared for food and feed purposes. Also, sublethal 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 sublethal 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.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.24559261

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Conflict of interest

The Netherlands Ministry of Economic Affairs had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. With the exception of Proti-Farm R&D, BV, the commercial entities in the consortium had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Proti-Farm R&D, BV provided in-kind contribution to the project primarily in the form of labour costs for author M.W.B., who assisted in the execution of experiments, which were performed at the premises of that company under supervision of the corresponding author, and for reviewing and editing the manuscript prior to submission. Proti-Farm R&D, BV otherwise had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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