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# The development of the solitary bee *Osmia bicornis* is affected by some insecticide agrochemicals at environmentally relevant concentrations

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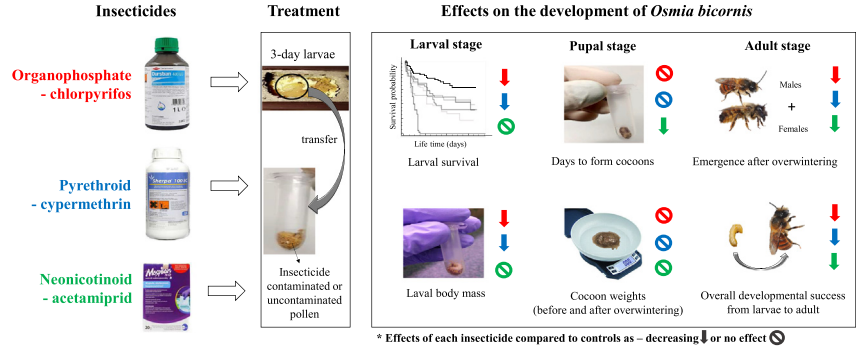
## HIGHLIGHTS

- Effects of agrochemicals on *Osmia bicornis* development were tested via pollen feeding.
- Chlorpyrifos and cypermethrin based agrochemicals decreased larval survival.
- Acetamiprid-agrochemical fed larvae started to spin cocoons earlier than controls.
- Bees development was affected even at field relevant concentrations of insecticides.
- Current risk assessment and pesticide usage regulations need to be revised.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Solitary bees provide essential pollination services for many arable crops, but are prone to global decline. Agricultural intensification, which is connected with pesticide usage, is among major threats to bees and, thus, to the food security and ecosystem stability. As it may not be possible to cease pesticide usage currently because of the growing demand for food, it is crucial to understand the pesticide toxicities to bees for better protection of pollinator populations. The majority of studies have focused on social bees, and those on solitary bees studied effects of adult exposure, whereas these bees are also likely to be exposed as larvae via the consumption of contaminated pollen. Here, the effects of three commonly used insecticide-based plant protection products on the development of the solitary bee, *Osmia bicornis* (red mason bee), were studied by exposing larvae to insecticide-contaminated multifloral pollen. The tested insecticides were: Dursban480EC, containing the organophosphate chlorpyrifos (CHP), Sherpa100EC, containing the pyrethroid cypermethrin (CYP), and Mospilan20SP with the neonicotinoid acetamiprid (ACT). When compared to the control larvae fed with uncontaminated-pollen, both CHP and CYP significantly reduced the *O. bicornis* larval survival and their body mass at all tested concentrations. In contrast, ACT did not affect either larval survival or body mass, but the length of larval stage to cocoon formation was significantly shortened compared to controls. None of studied insecticides affected the mass of cocooned individuals. However, at least 80% of individuals exposed to any of the tested insecticides died before reaching the adult stage, whereas 43% of the controls emerged successfully after overwintering. Although no clear monotonic dose-response relationships were found, our study showed that at least some insecticide formulations affect the development of *O. bicornis* even at concentrations actually found in pollen in the field, indicating an urgent need for revising current pesticide usage recommendations.



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## 1. Introduction

Bees are the dominant group of pollinators providing insect pollination services to several crops (Klein et al., 2007), influencing about 35% of global agricultural land, with crop vulnerability to the loss of pollinators being more than 12% in regions of Central and Eastern Asia and 11% each in Europe and North America (Potts et al., 2010). In revenue, the global economic value of crop pollination services by bees range from USD 235 to 577 billion per year (FAO, 2019). Maintaining bee biodiversity and abundance is, therefore, the key factor for global food security because without pollinators many plants will stop producing fruits, which in extreme events can bring famine, at least on a local scale, and in more moderate cases will lead to dramatic increases in food production costs, and as a result – in food prices. Although a significant proportion of global crop pollination demands relies on the managed honey bees of *Apis* genus, the solitary bees serve as the principal or only pollinators for several fruit and nut crops in many temperate and tropical areas (Felicioli and Pinzauti, 2008). Recently, the importance of solitary bees for pollination was underlined in many crops across the world: in Europe mainly for rapeseed cultivations (*Brassica napus*) (Holzschuh et al., 2013) and strawberries (Herrmann et al., 2019), in North America for sunflowers (Mallinger et al., 2019), in the Asia-Pacific for many orchids (Son et al., 2019) and in Africa for coffee plantations (Chain-Guadarrama et al., 2019). Despite their high economic and ecological significance, there has been increasing evidence that the pollinator populations have been declining significantly over the last few decades (Powney et al., 2019; Bartomeus et al., 2013; Cameron et al., 2011; Potts et al., 2010), consequently generating negative impacts on the socio-economic development of mankind (Ritten et al., 2018). Many different factors, including agriculture intensification, is blamed for this phenomenon (Goulson et al., 2015; Kennedy et al., 2013; Sandrock et al., 2014; Potts et al., 2010). It has particularly been shown that the intensive and improper agricultural usage of pesticides, mainly insecticides, cause huge population damages among bee pollinators in the long-term (Brittain and Potts, 2011). However, as it may not be possible to stop using pesticides at this time due to the ever growing demand for food, it is crucial that we understand their effects and use them in a way that does not jeopardise pollinator populations.

Because of their biological and morphological differences and the lack of social lifestyle, solitary bees may be affected by pesticides differently than social *Apis* bees (Uhl and Brühl, 2019; Brittain and Potts, 2011), and yet they have received much less attention in pesticide monitoring and regulation (Eeraerts et al., 2020; Blacquière et al., 2012). In effect, the European Food Safety Agency (EFSA) suggested to include the red mason bee, *Osmia bicornis*, as a model organism for non-*Apis* solitary bees in the pesticide risk assessment scheme (EFSA, 2013). However, the scarcity of information about the direct or indirect effects of pesticides and robust toxicity test methods for this species limits its use in current risk assessment. In particular, the possible detrimental effects of insecticides during the developmental stages of *O. bicornis* are pivotal for understanding the long-term effects on population dynamics. To date, a few studies have tested the effects of pesticides using solitary bee larvae of the genus *Osmia* spp. (Dharampal et al., 2018; Nicholls et al., 2017; Sgolastra et al., 2015; Tesoriero et al., 2003), but differences among the different test protocols and endpoints measured make the results of those studies difficult to compare. Therefore, further standardisation of the oral toxicity test protocol is necessary to identify representative endpoints for both lethal and sublethal effects and develop a reliable toxicity protocol for solitary bee larvae (Eeraerts et al., 2020).

In general, the red mason bees *O. bicornis* are relatively easy to propagate in artificial nests. During spring, female *O. bicornis*, after insemination, start colonising nest tubes and build 5 to 34 cells. Each cell is stocked with ca. 100–300 mg of collected pollen mixed with a small amount of nectar and a single egg is laid directly on the pollen (Giejdasz et al., 2016). Some studies found a positive effect of proximity of rapeseed cultivations on the number of nesting *O. bicornis* (Holzschuh et al., 2013; Jauker et al., 2012), but such mass-flowering crops, which can provide reliable, although short-lived, pollen and nectar resources for wild pollinators, are usually sprayed with a range of plant protection products. Residues of insecticides were found not only in the pollen and nectar of the sprayed crops, but also in wildflowers growing in the field margins (Botías et al., 2015). Mullin et al. (2010) found as many as 98 pesticides and metabolites in honey bee-collected pollen near agricultural fields in the US and Canada, including organophosphates, pyrethroids, neonicotinoid insecticides and azol-fungicides. Similarly, a survey of Italian honey bee-collected pollen revealed widespread contamination by agricultural pesticides (Tosi et al., 2018) and Piechowicz et al. (2018) demonstrated that active ingredients of plant protection products, such as chlorpyrifos in Dursban, are transferred from soil to flowers to bee hives. By bringing pesticide-contaminated pollen to their nests, which then serves as a sole food source to the developing larvae, *O. bicornis* larvae can be exposed to a range of pesticides orally and trans-dermally. Moreover, because red mason bees collect food for one larvae over only 1 to 2 days, then lay an egg and the larva starts to eat pollen immediately after hatching, usually within ca. 7 days, the concentrations of pesticides in the maternal-provided pollen are expected to be high during the first few days of larval feeding and could conceivably harm the developing bees (Sgolastra et al., 2019). Studies on honey bees showed that some insecticide residues present in bee-collected pollen exert greater toxicity to larvae than to adult bees (Zhu et al., 2014; Heylen et al., 2011). The insecticides toxic to bee larvae include, for example, encapsulated organophosphates, systemic neonicotinoids (Dai et al., 2017; Rortais et al., 2005) and pyrethroid formulations (Yang et al., 2019). Consumption of such insecticide-laden pollen may not only cause high mortality among larvae, but also influence their development to pupae and imago, as was shown for the honey bee exposed to thiamethoxam (Tavares et al., 2019).

Furthermore, the sensitivity of bees to a particular insecticide may differ between the active ingredient and typical agrochemical formulations due to added materials (Mullin, 2015). These added adjuvants/co-formulants include surfactants, penetrant enhancers, activators, spreaders, stickers, wetting agents, buffers, antifoaming agents, drift retardants, etc., and are being used to facilitate tank-mixing and achieve high efficacy of pesticides towards the targeted pests, but inadvertently affect also non-target beneficiary organisms such as bees (Mullin et al., 2015). These adjuvants in the commercial formulations normally potentiate the toxicity of active ingredients by enhancing their penetration ability and systemic movement (Mullin, 2015). However, as stressed by Benuszak et al. (2017), the majority of the published studies on toxicity of pesticides to honey bees had only tested their active ingredients in pure form rather than agrochemical formulations that are actually used in real-field scenarios. Hence, in order to understand the real-world effects of pesticides on bee pollinators and other non-target organisms, toxicity testing should be performed on whole agrochemicals (commercial formulations) rather than on sole active ingredients. Although some regulatory agencies, mostly in the EU and US, require testing whole formulations, these tests are restricted to honey bees, while toxicity of commercial formulations to non-*Apis* bees remains unknown.

This study was aimed at finding out whether insecticide formulations at concentrations of active ingredients (a.i.) in pollen similar to the highest actually measured in pollen in agricultural fields (see Mullin et al., 2010) affect the development of *O. bicornis* from larval stage to the emerged adults. We tested the effects of three agrochemical formulations representing different, commonly used, insecticide types, namely Dursban 480 EC containing the organophosphate chlorpyrifos, Sherpa 100 EC with the pyrethroid cypermethrin, and Mospilan 20 SP with the neonicotinoid acetamiprid, on the development of *O. bicornis* from larvae to adult. The toxic effects of the chronic exposure of larvae to insecticides can be manifested as increased larval mortality, decreased body mass, altered larval development (e.g., time to pupation),

increased overwintering mortality, prolonged time to emergence of adults or overall failure to emerge. All of the above-mentioned endpoints were studied.

## 2. Materials and methods

### 2.1. Rearing of O. bicornis larvae

Cocoons of the solitary red mason bee, *O. bicornis* (previously known as *Osmia rufa,* Hymenoptera: Megachilidae), were purchased from a local supplier (BioDar, Poland) in February 2018 and stored at 4 °C until use. Adult red mason bees were reared from the cocoons in disposable nest cases installed in the apiary of the Institute of Environmental Sciences, Jagiellonian University in Kraków, Poland, from April to May 2018. In total, 4000 cocoons were placed into 55 nesting cases (four wooden nesting blocks, each containing a set of 13 or 14 nesting cases and 1000 cocoons placed on top of each block). Each nesting case consisted of 20 nesting tubes made of polystyrene, opened at one end only, 20 cm in length and 6.5 × 8.5 mm internal dimensions of each tube (Fig. S1a in Supplementary Materials). The wooden racks with the nesting cases helped in easy handling and transportation to the laboratory. Each nesting tube was numbered consecutively for the record. Once the temperatures rose above 15 °C, the bees started to emerge from the cocoons, first the males followed by the females, and were observed to mate near the nesting cases. Few days later the females started to collect pollen and laid eggs (Fig. S1b). The date of each laid egg and of larvae hatching was recorded every day together with its position in the nest. Based on our preliminary observations of the susceptibility of *O. bicornis* larvae to mechanical stress during transfer from native pollen to pollen in experimental tubes, 3-day old larvae were found to well withstand the procedure, hence we decided to use exclusively larvae of that age in the experiment.

### 2.2. Preparation of insecticide contaminated pollen

Three commercially available plant protection products that are commonly used by farmers in Poland were used for the study: Dursban 480 EC (44.86% chlorpyrifos, CHP, as a.i.), Sherpa 100 EC (10.76% cypermethrin, CYP, as a.i.), and Mospilan 20 SP (20% acetamiprid, ACT, as a.i.). The percent a.i. values were used to calculate concentrations of the active ingredients in solutions to be mixed with the required amount of pollen for treatments.

Pellets of multi-floral pollen collected by honey bees were purchased from a local beekeeper's store (Pszczelarz Kozacki, Kozaki, Poland). The pollen pellets were fine powdered using a kitchen grinder and stored in an airtight glass jar at 4 °C until use. The average water content in the powdered pollen was 14%. Hence, an additional 10% moistening of the pollen was required to yield a preparation comparable to the pollen collected by wild *Osmia* bees (~20% water content) (Dharampal et al., 2018).

Treatment solutions of each insecticide were prepared with 5-fold dilution factor, starting from the highest concentrations used to contaminate the experimental pollen: 48 μg/mL for CHP, 50 μg/mL for CYP and 10 μg/mL for ACT in distilled water. For each pollen treatment preparation, 1.5 mL of test insecticide solution was added to 15 g of powdered pollen in a glass beaker and mixed thoroughly by continuous stirring with a sterile spatula for at least 15 min to assure uniformity. The concentrations of insecticides were expressed per wet weight of the pollen (i.e., ≈ 16.5 g which is the final mass after adding 1.5 mL test solution). Pollen mixed with only distilled water as solvent in same manner as described above was used for the control treatment. A total of 16 treatments, including the control, were used with the following nominal insecticide concentrations expressed as ng of a.i. per g of the total wet pollen: for CHP in Dursban – 7, 35, 175, 873 and 4364 ng/g, for CYP in Sherpa – 7, 36, 182, 909 and 4545 ng/g, and for ACT in Mospilan – 1, 7, 36, 182 and 909 ng/g. The range of treatment concentrations selected for this study covered the maximum observed concentrations (i.e., 830 ng/g for CHP, 49 ng/g for CYP and 134 ng/g for ACT) as well as the 90th centiles (i.e. 140 ng/g for CHP, 28 ng/g for CYP and 101 ng/g for ACT) in pollen samples from North American honey bee colonies (Mullin et al., 2010). After mixing the pollen with solutions of insecticides, each preparation of contaminated pollen was transferred to individual 2 mL Eppendorf tubes, 201 ± 2.6 mg per tube (Mean ± SD) (but see Eeraerts et al. (2020) for mass of pollen provisions suggested for eggs and first instar larvae), on ice and stored at −20 °C for later treatments. Pesticides in the control pollen was screened for residues of 466 different substances (Table S1 in Supplementary Materials), including CHP, CYP and ACT, and the concentrations of active ingredients in the contaminated pollen were confirmed in selected samples used for the experiment. The chemical analyses were done by the certified external contractor – the Regional Experimental Station of the Institute of Plant Protection, National Research Institute in Białystok, Poland, using LC-MS/MS or GS-MS/MS techniques with limit of quantification of 0.1 ng/g for CHP and ACT and 5 ng/g for CYP. The results of chemical analysis are presented in Table 1a and b. The measured concentrations were within 20% of the nominal values for all insecticidal treatments (Table 1a).

### 2.3. Transfer of 3-day-old larvae to contaminated pollen and monitoring

Each day, prior to transferring the larvae to the treatment, the pre-prepared Eppendorf tubes supplied with insecticide-contaminated pollen (or water for controls) were warmed to room temperature. Wooden blocks containing selected nesting cases with larvae were carefully moved to the lab from the apiary. To cause as limited disturbance as possible to the freshly hatched bee larvae during repeated transferring of nesting cases to the lab, three days were chosen for starting experiments when a maximum number of larvae were expected to turn 3-days old. Each three-day old *O. bicornis* larva was then carefully transferred onto the pollen in the Eppendorf tubes using soft forceps (one larva per tube, $n = 50$ for each insecticide concentration and $n = 120$ for the control) (Fig. S1c – d). The higher number of control larvae was used because all insecticidal treatments were compared against the control, so precise estimation of all measured endpoints in the control treatment was crucial for detecting insecticide effects. Besides, it has been reported that natural mortality of *O. bicornis* larvae can be high due to unknown reasons (Nicholls et al., 2017).

During the transfer, larvae from the same nest case were randomly assigned across the treatments ensuring, however, that not all larvae from either end of the nesting tube were assigned to the same treatment, eliminating thus the potential genetic and/or sex biases. Usually fertilised eggs which produce female progeny are laid on larger provisions in the innermost cells within a nesting tube, whereas unfertilised eggs producing male progeny are allocated smaller provisions in the outermost cells (Bosch and Vicens, 2002). Hence, although by selecting exclusively larvae from cells located at the very ends of nesting tubes the chances of obtaining giving sex are significantly higher that if sampled randomly, the actual male-to-female ratio remains unknown, as the distribution of both sexes in the nest tube is not always the rule. Raw (1972), for example, found that half of the 74 tubes of *Osmia rufa* (=*O. bicornis*) examined contained bees of both sexes and half contained only one sex. The experimental tubes with larvae were kept in the climatic chamber at 20 ± 2 °C, 70 ± 5% relative humidity (RH) in complete darkness to facilitate larval and pupal development (Nicholls et al., 2017), for 30 days, and later at reduced RH of 30% to avoid fungal growth on the formed cocoons. Larval survival was visually monitored every day. Each 18-day larvae (i.e. fed with insecticide-contaminated or control pollen for 15 days) was weighed to the nearest 0.001 g (WPA180/k; Radwag, Radom, Poland). Later, the time of starting of cocoon formation was recorded for each larva and each cocoon was weighed after 170 days since the start day of exposure. Because bees are sensitive especially to the pre-wintering and wintering

**Table 1**
Concentrations of active substances in the pollen used in the studies on pesticide effects on the development of *Osmia bicornis* larvae: a. nominal and measured concentrations of the three experimental insecticides in selected samples; b. residues of pesticides found in control pollen (LOQ – Limit of Quantification).

| Product | Active substance detected | Nominal concentration (ng/g pollen) | Measured concentration (ng/g pollen) | LOQ | Technique |
|---|---|---|---|---|---|
| | | | Mean ± SD | ng/g | |
| a. Values for three plant protection products tested | | | | | |
| Dursban 480 EC | Chlorpyrifos-ethyl | 174 | 184 ± 9 | 0.1 | LC-MS/MS |
| | | 4364 | 3655 ± 139 | | |
| Sherpa 100 EC | Cypermethrin | 182 | 146 ± 4 | 5 | LC-MS/MS |
| | | 4545 | 4248 ± 196 | | |
| Mospilan 20 SP | Acetamiprid | 36 | 31 ± 2 | 0.1 | LC-MS/MS |
| | | 909 | 900 ± 45 | | |
| b. Values for control pollen[a] screened for 466 chemical substances (see supplementary Table S1 for screening details) | | | | | |
| Control pollen (with water) | Acetamiprid | _ | 2.7 ± 0.1 | 0.1 | LC-MS/MS |
| | Azoxystrobine | _ | 19.6 ± 0.7 | 1 | GC–MS/MS |
| | Carbendazim | _ | 12.0 ± 0.4 | 0.1 | LC-MS/MS |
| | Chlorpyrifos | _ | 2.8 ± 0.1 | 0.1 | GC–MS/MS |
| | Difenoconazole | _ | 1.6 ± 0.3 | 0.01 | GC–MS/MS |
| | Hexachlorobenzene | _ | 3.1 ± 0.2 | 1 | GC–MS/MS |
| | Metolachlor | _ | 1.1 ± 0.0 | 0.1 | GC–MS/MS |
| | Thiacloprid | _ | 15.7 ± 0.5 | 0.1 | LC-MS/MS |

[a] Control pollen was mixed with water as solvent in the same procedure as used for the insecticidal treatments.

temperatures (Bosch and Kemp, 2004), a gradual decrease in the temperature was used for reaching the overwintering conditions in our study. In October 2018, the cocoons in all treatments were prepared for overwintering by decreasing the temperature by 2 °C per week to reach 4 °C in December 2018. In March 2019, after winter incubation, the cocoons were weighed again, moved to the climatic chamber set at 20 ± 2 °C, 70 ± 5% RH and 16:8 h L:D and observed daily for the emergence of adults. Raw data with the dates of larval hatching, exposure, mortality, cocoon formation, overwintering, emergence and identified sex for each larval treatment were provided in a separate MS Excel file as Supplementary Material 2.

### 2.4. Statistical analysis

All statistical analyses for the studied endpoints started with an overall test across all treatments for significant treatment effect. If significant effect on an endpoint was detected, each insecticide was tested individually along with control group. In the case of significant effect of an insecticide on larvae survival, pair-wise comparisons of survival curves were performed within this insecticide plus control. For other endpoints the means were separated using Fisher's least significant difference (LSD) procedure. Significance level for all statistical tests was set at $p \leq 0.05$.

Survival curves of larvae were established through Kaplan-Meier estimator function by censoring the data to the start day of cocoon formation. All larvae which pupated successfully were considered as live (census 1). However, some larvae failed to form proper cocoons (see Table 2). As described above, the survivorship was first compared among all treatments, followed by separate tests for each insecticide while including the controls. Log-rank test was used to compare the survival probabilities across treatment groups at 95% confidence interval. In most of the treatments mortality of the larvae during the experiment was high enough to estimate median lethal times ($LT_{50}$). All larval survival analyses were performed in Statgraphics™ Centurion XVII.

**Table 2**
Sample size of *Osmia bicornis* reared on pollen provisions spiked with different concentrations of three insecticides: Dursban 480 EC, Sherpa 100 EC and Mospilan 20 SP at particular developmental stage – as larvae, pupae and emerged adults after overwintering, and percent survival with respect to the previous developmental stages. N – sample size, *m* – males and *f* – females.

| Treatment | Concentration of active ingredient in pollen (ng/g) | N larvae treated (N initial) | *Larval stage* | | *Pupal stage* | | | | *Adults stage* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | N larvae survived | % larvae survived vs N initial | N cocoons | | % formed cocoons vs N initial | % formed cocoons vs N larvae survived | N emerged | | % adults vs N initial | % adults vs N larvae survived | % adults vs N formed cocoons |
| | | | | | formed | failed | | | *m* | *f* | | | |
| Control (distilled water) | 0 | 120 | 83 | 69 | 76 | 7 | 63 | 92 | 30 | 21 | 43 | 52 | 67 |
| Chlorpyrifos (Dursban 480 EC) | 7 | 50 | 14 | 28 | 8 | 6 | 16 | 57 | 2 | 1 | 6 | 43 | 38 |
| | 35 | 50 | 23 | 46 | 13 | 10 | 26 | 57 | 7 | 0 | 14 | 61 | 54 |
| | 175 | 50 | 17 | 34 | 10 | 7 | 20 | 59 | 3 | 0 | 6 | 35 | 30 |
| | 873 | 50 | 23 | 46 | 17 | 6 | 34 | 74 | 6 | 1 | 14 | 61 | 41 |
| | 4364 | 50 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | | | |
| Cypermethrin (Sherpa 100 EC) | 7 | 50 | 13 | 26 | 9 | 4 | 18 | 69 | 2 | 0 | 4 | 31 | 22 |
| | 36 | 50 | 26 | 52 | 19 | 7 | 38 | 73 | 4 | 0 | 8 | 31 | 21 |
| | 182 | 50 | 17 | 34 | 12 | 5 | 24 | 71 | 3 | 1 | 8 | 47 | 33 |
| | 909 | 50 | 18 | 36 | 12 | 6 | 24 | 67 | 4 | 2 | 12 | 67 | 50 |
| | 4545 | 50 | 16 | 32 | 14 | 2 | 28 | 88 | 3 | 0 | 6 | 38 | 21 |
| Acetamiprid (Mospilan 20 SP) | 1 | 50 | 32 | 64 | 22 | 10 | 44 | 69 | 5 | 1 | 12 | 38 | 27 |
| | 7 | 50 | 30 | 60 | 26 | 4 | 52 | 87 | 5 | 0 | 10 | 33 | 19 |
| | 36 | 50 | 28 | 56 | 19 | 9 | 38 | 68 | 5 | 3 | 16 | 57 | 42 |
| | 182 | 50 | 31 | 62 | 24 | 7 | 48 | 77 | 3 | 2 | 10 | 32 | 21 |
| | 909 | 50 | 35 | 70 | 23 | 12 | 46 | 66 | 6 | 4 | 20 | 57 | 43 |

Developmental differences among treatments were assessed in terms of 18-day larval body mass, days required to start cocoon formation, and cocoon mass before and after overwintering. Larval body mass and cocoon mass were first checked for normality of distribution via inspection of residuals vs. fitted and normal Q–Q plots combined with Shapiro-Wilk statistic, and for homogeneity of variance by Levene test. If the assumptions were not met, log-transformed data were used for further analysis. The overall treatment effect for all 16 treatments, either in the larval body mass or in the cocoon mass, was tested using one-way ANOVA. The effects on development time of larvae to pupae, expressed as number of days required to start cocoon formation, were assessed by generalised linear model (GLM) with Poisson distribution linked to natural logarithmic function because it not only considered count data but also the time to start cocoon formation. Individuals failing to form cocoons or exhibiting other uncertain behaviours were excluded from statistical analysis except for 18-day larval body mass (the exact number of failed individuals for each treatment is presented in Table 2).

One-way ANOVA was used to test the overwintering effects on cocoon weight loss (expressed as mass change index, i.e. cocoon weight before overwintering minus after overwintering divided by the before overwintering) across treatments. The overall developmental success of bees since starting the experiment till adult emergence was analysed by the generalised linear model as a binomial binary function (died vs. emerged) – binomial GLM. All statistical tests except survival curves were performed in R-3.5.0 (R Foundation for Statistical Computing, Vienna, Austria).

## 3. Results

### 3.1. Survival of O. bicornis after exposure to insecticides

In total, 69% (83 out of 120) of control *O. bicornis* larvae survived till cocoon formation, from which ca. 92% (76 out of 83) formed cocoons and 67% (51 out of 76) reached the adult stage. The number of individuals which survived till 18-day larvae, formed pupae and emerged together with survival percentage to the next stage in each treatment are presented in Table 2. The 100% mortality of larvae was found at the highest CHP concentration in pollen (4364 ng/g). In all other pesticide treatments, survival of the larvae till cocoon formation was 26–70%, but only 16–52% of treated larvae were able to actually form cocoons (63% in control), and 4–20% of individuals emerged successfully (43% in the control). A total of 100 larvae out of 406 which survived till cocoon formation were not able to spin cocoons properly and thereby pupated outside of the cocoons (Table 2) and eventually died, thus, these individuals were excluded from some statistical analysis (i.e. analysis of days required to start cocoon formation, analysis related to cocoon mass). Such cases accounted for 8% in the control treatments (7 out of 83 larvae which survived till cocoon formation did not spin cocoons properly) and for 12–43% in the insecticidal treatment groups, with the highest percent of failed cocoons found for Dursban-CHP treatments (see Table 2).

The survival curves of larvae differed significantly (log rank test: $\chi^2 = 139.9$, df = 15, $p < 0.0001$) between 16 treatments and were analysed successively within each insecticide while including the controls. Significant differences among treatments were found for Dursban-CHP (log-rank test: df = 5, $\chi^2 = 107.0$, $p < 0.0001$) and Sherpa-CYP (log-rank test: df = 5, $\chi^2 = 35.8$, $p < 0.0001$). The larval survival declined rapidly in all Dursban-CHP and Sherpa-CYP concentrations with respect to controls (Fig. 1a and b). The pairwise comparisons of each treatment against the control for Dursban-CHP and Sherpa-CYP and the median survival times ($LT_{50}$) are presented in Table 3. In contrast, there was no significant effect of Mospilan-ACT on larval survival (log rank: $\chi^2 = 4.01$, df = 5, $p = 0.55$) (Fig. 1c) with 68–72% survival till 18 days larvae and 56–70% survival till cocoon formation in all Mospilan-ACT treatments. However, in the Mospilan-ACT-treated bees increased mortality occurred as late as in pupal or cocooned imago stage, resulting in 38–52% formed cocoons and only 10–20% of emerged adults (see Table 2 for percent of *O. bicornis* survival with respect to the developmental stages).

### 3.2. Effects of insecticides on larval body mass, cocoon formation and cocoon weight

The overall significant differences among all treatments were observed for all analysed parameters of *O. bicornis* development, namely for $\log_{10}$-transformed 18-day larval body mass (ANOVA: $F_{14,515} = 3.74$, $p < 0.0001$) (Fig. 2), days to start cocoon formation (generalised linear model GLM: estimate±SE = 3.61 ± 0.02, z = 188.1, $Pr(>|z|) < 0.0001$; Table S2) (Fig. 3), and cocoon weights either before or after overwintering (ANOVA: $F_{14,274} = 1.89$, $p = 0.03$ for both log-transformed weights). After overwintering, the weight loss of cocoons was uniform across all treatments (ANOVA: $F_{14,273} = 0.49$, $p = 0.93$ for log-transformed mass change index).

The body mass of larvae fed CHP-contaminated pollen was reduced (ANOVA: $F_{4,215} = 4.05$, $p = 0.0035$) and differed significantly from

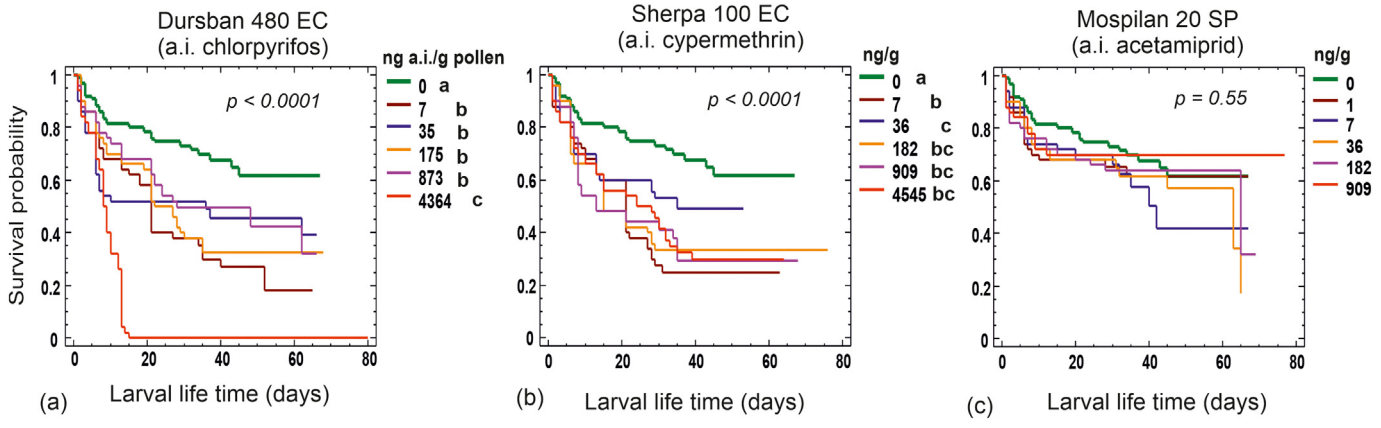


**Fig. 1.** Survival curves of *O. bicornis* larvae after exposure to three insecticides: (a) Dursban 480 EC – a.i. chlorpyrifos, (b) Sherpa 100 EC – a.i. cypermethrin, and (c) Mospilan 20 SP – a.i. acetamiprid. Sample sizes are n = 50 per each insecticide treatment and n = 120 for the control. Concentrations are expressed as ng of a.i. per g of pollen. Data was censored at the start day of cocoon formation for statistical analysis. Concentrations marked with the different lower-case letters indicate significant differences at p ≤ 0.05 in log-rank test. Treatment concentrations for each pesticide cover the maximum observed concentrations (i.e. 830 ng/g for CHP, 49 ng/g for CYP and 134 ng/g for ACT) as well as the 90th centiles (140 ng/g for chlorpyrifos, 28 ng/g for cypermethrin and 101 ng/g for acetamiprid) in the bee collected pollen in agricultural fields reported by Mullin et al. (2010). (Preference for color: in both print and online versions).

**Table 3**
Median lethal times ($LT_{50}$s) with standard error (SE) for *Osmia bicornis* larvae exposed to different concentrations of insecticides in pollen and the results of log-rank comparison of survival curves for each treatment against the control ($\chi^2$ and significance level $p$).

| Treatment | Nominal concentration in pollen (ng/g) | $LT_{50} \pm$ SE (days) | χ2 (log-rank) | *p*-Value compared to control |
|---|---|---|---|---|
| Control | 0 | >80 | | |
| Chlorpyrifos (Dursban 480 EC) | 7 | 21 ± 1.1 | 21.96 | <0.0001 |
| | 35 | 36 ± 23.8 | 8.56 | 0.003 |
| | 175 | 22 ± 2.9 | 15.85 | <0.0001 |
| | 873 | 28 ± 12.0 | 7.19 | 0.007 |
| | 4364 | 8 ± 0.6 | 103.74 | <0.0001 |
| Cypermethrin | 7 | 21 ± 2.1 | 27.09 | <0.0001 |
| (Sherpa 100 EC) | 36 | 35 ± ne | 5.36 | 0.02 |
| | 182 | 15 ± 2.8 | 18.83 | <0.0001 |
| | 909 | 13 ± 6.6 | 17.80 | <0.0001 |
| | 4545 | 24. ± 7.2 | 18.27 | <0.0001 |

ne – Not estimated.

control larvae at all concentrations tested. No differences were found, however, between Dursban-CHP treatments, despite the broad range of concentrations (7–873 ng/g) used (Fig. 2a). No effect of Dursban-CHP was found for time to start cocoon formation (GLM: $z = -1.6, Pr(>|z|) = 0.12$; Fig. 3a) and for the cocoon mass either before (ANOVA: $F_{4,112} = 1.33$, $p = 0.26$) or after overwintering (ANOVA: $F_{4,112} = 1.12$, $p = 0.35$). However, it must be noted that only 16–34% of the treated larvae were able to form cocoons in all Dursban-CHP treatments except for CHP-4364 in which all larvae died, while 63% of the control larvae pupated successfully (Table 2).

Similarly, Sherpa-CYP also reduced larval body mass in all treatments in comparison to the controls (ANOVA: $F_{5,226} = 5.24$, $p = 0.0001$), with no concentration dependency (Fig. 2b). No effects were found for the time to cocoon formation (GLM: $z = -1.9$, $Pr(>|z|) = 0.11$; Fig. 3b) and for cocoon weights either before (ANOVA: $F_{5,130} = 1.68$, $p = 0.14$) or after overwintering ($F_{5,130} = 1.52$, $p = 0.19$). There was not any trend in the percentage of larvae which pupated successfully (18–40%) after exposure to increasing concentrations of Sherpa-CYP in pollen (Table 2).

In contrast, *O. bicornis* larvae which received Mospilan-ACT contaminated pollen were not affected either in larval body mass (ANOVA: $F_{5,264} = 0.99$, $p = 0.43$; Fig. 2c) or in cocoon weights before (ANOVA: $F_{5,176} = 0.79$, $p = 0.56$) and after overwintering ($F_{5,176} = 1.05$, $p = 0.39$). However, the time to cocoon formation was considerably shorter in all Mospilan-ACT treatments than in the controls (GLM: $Pr(>|z|) < 0.0001$; Table S2) (Fig. 3c). The percentage of treated larvae which formed cocoons successfully was between 38 and 52% (Table 2).

### 3.3. Overall developmental success

The overall bee developmental success is defined in this study as survival of individuals from the beginning of experiment (3-day old larvae) to emerging successfully from cocoons in the next spring after overwintering. Most of the insecticides treated larvae (≥80%) did not reach the adult stage, whereas 43% of the control larvae developed successfully into adults (Table 2). The overall developmental success of individuals, i.e. emerging as adults in the

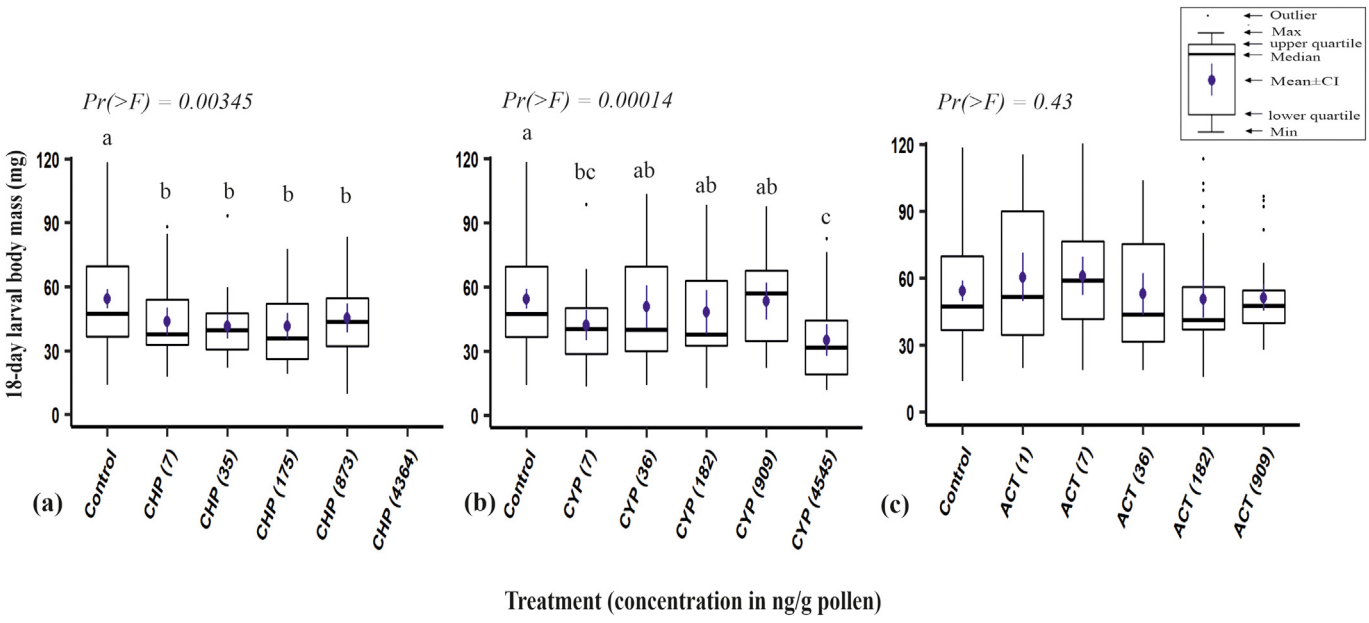


**Fig. 2.** Body mass of *Osmia bicornis* larvae fed for 18 days with pollen spiked with different concentrations of three insecticides: Control – larvae fed pollen mixed with only water, CHP – chlorpyrifos in Dursban 480 EC, CYP – cypermethrin in Sherpa 100 EC and ACT – acetamiprid in Mospilan 20 SP; nominal concentrations in brackets. No larvae survived by 18-days of their age in the highest chlorpyrifos concentration (4364 ng/g). One-way ANOVA was performed on log10-transformed body mass for all treatments together ($p < 0.0001$) and later individually for each insecticide while including controls. Pairwise comparisons were done for CHP and CYP treatments since in both cases significant effect at $p \le 0.05$ was detected. Different lowercase letters indicate significant differences (LSD) between treatments at $p \le 0.05$.

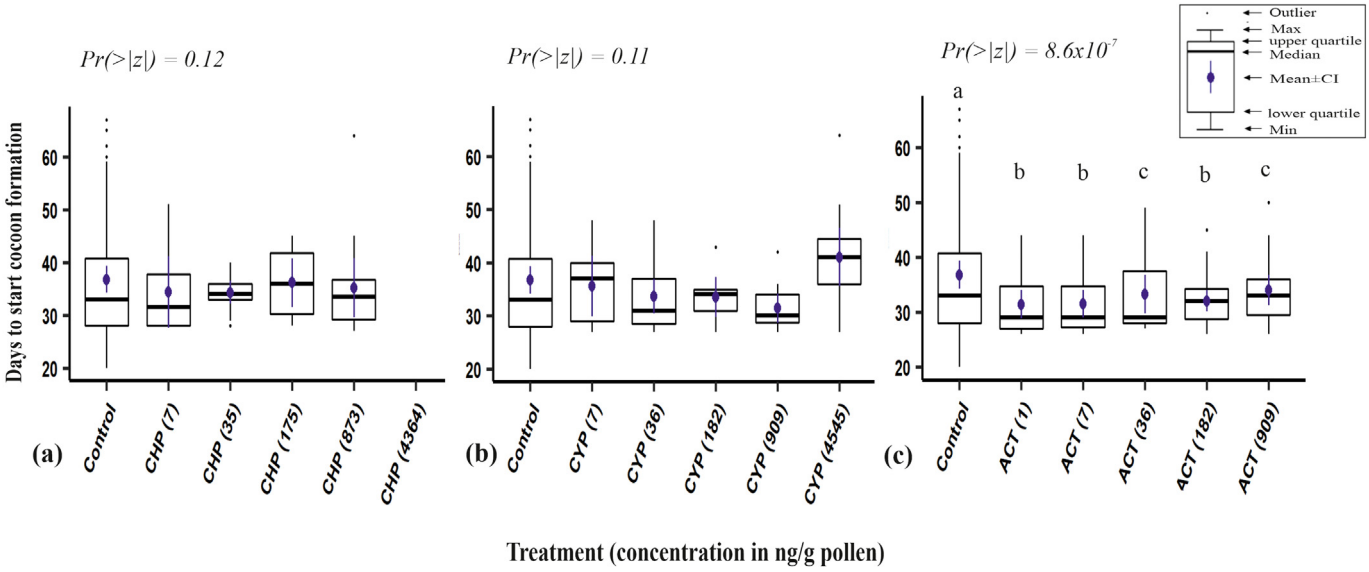


**Fig. 3.** Time (days) to start cocoon formation by *Osmia bicornis* larvae fed pollen spiked with different concentrations of three insecticides: Control – larvae fed pollen mixed with only water, CHP – chlorpyrifos in Dursban 480 EC, CYP – cypermethrin in Sherpa 100 EC and ACT – acetamiprid in Mospilan 20 SP; nominal concentrations in brackets. No larvae survived until cocoon formation at the highest chlorpyrifos concentration (4364 ng/g). Generalised linear model (GLM) with Poisson function was used to analyse the differences among treatments altogether ($Pr(>|z| < 0.0001$) and individually for each insecticide while including the control treatment. Pairwise comparisons were done for ACT treatments since the differences were significant at $p \leq 0.05$. Different lowercase letters indicate significant differences (LSD) between treatments at $p \leq 0.05$.

next spring, was significantly lower in all insecticidal treatments in comparison with controls (GLM binomial model as emerged versus not emerged: $z = -5.92$, $Pr(>|z|) < 0.0001$ for Dursban-CHP; $z = -7.19$, $Pr(>|z|) < 0.0001$ for Sherpa-CYP; $z = -7.28$, $Pr(>|z|) < 0.0001$ for Mospilan-ACT; $N = 120$ for controls and $N = 50$ for each insecticidal treatment), with no differences between treatments with pollen contaminated with different insecticides or at different concentrations (see Table S3 for comparisons among insecticides and between concentrations within each insecticide).

## 4. Discussion

Our experiment showed that chronic dietary exposure of *O. bicornis* larvae to chlorpyrifos-containing Dursban, cypermethrin-Sherpa or acetamiprid-Mospilan, even at field-realistic concentrations, had a significant impact on survivorship from larvae to adults. We confirmed that not only lethal but also sublethal effects during larval development (e.g., the duration of development from second instar to cocoon spinning initiation, body mass, weight loss during hibernation) can be expected as negative effects of exposure to insecticides (Eeraerts et al., 2020).

Dursban-CHP and Sherpa-CYP in our artificially contaminated pollen affected survival of *O. bicornis* larvae by the time of pupation even at the lowest tested concentrations (7 ng/g of pollen) against the controls. In contrast, neonicotinoid-based Mospilan (with acetamiprid as an active ingredient) did not cause significant mortality during larval stage in comparison with the control even at high concentrations, but eventually affected *O. bicornis* development into adults, mostly via the increased pupae mortality (Table 2). The experiment clearly demonstrated that Dursban and Sherpa had more immediate effect by killing larvae, while Mospilan delayed effects were visible at pupal stage, finally resulting in the similar net effect at the population level.

Chlorpyrifos affects acetylcholinesterase activity while cypermethrin disrupts voltage gated sodium channels, both damaging the transmission of neural signals. This eventually may result in either death or affect larval development once sufficient concentrations of insecticides and/or their metabolites have accumulated (Kadala et al., 2019). For instance, Zhu et al. (2014) reported high accumulative toxicity of chlorpyrifos at 1.5 μg/mL of larval diet in the honey bee, causing over 50% mortality in 6 days of exposure. Dai et al. (2017) validated the lethal toxicity of chlorpyrifos in *A. mellifera* larvae through contaminated diet and determined the 72 h $LD_{50}$ at 0.46 μg/larva. In our study, due to the over 50% larval mortality until cocoon formation even at lowest test concentrations of Dursban-chlorpyrifos and Sherpa-cypermethrin (7 ng/g pollen), substantial mortality (31%) of control larvae and the lack of monotonic concentration-response relationship, it was not possible to estimate median lethal concentrations of these insecticides. Yet, these lowest tested concentrations were about 125 and 5 times lower than the highest field concentrations reported for chlorpyrifos and cypermethrin in pollen, respectively (Mullin et al., 2010), indicating that these two pesticides can indeed affect negatively developing *O. bicornis* in agricultural areas (cf. Uhl et al., 2019). The lack significant decrease in survival of larvae fed Mospilan-acetamiprid contaminated pollen might suggest that Mospilan (and, perhaps, other acetamiprid-based plant protection products) are relatively safe for *O. bicornis*. Indeed, Brunet et al. (2005) found the rapid metabolism of acetamiprid in honey bees after oral exposure, with an elimination half-life as short as ca. 25 min in the whole honeybee. This was later confirmed with the acetamiprid related toxicity in other bee species (Dworzańska et al., 2020; Manjon et al., 2018). In our studies, however, the acetamiprid-based Mospilan caused delayed effects, resulting in a significant drop of adult emergence success in comparison with the control, apparently through high mortality during the pupal stage or overwintering adults. Similar to these results, Tavares et al. (2017) observed that upon sublethal oral exposure of *A. mellifera* to the neonicotinoid thiamethoxam, the survival rate of larvae was not affected but the pupal survival decreased significantly and thus the percentage of emerged bees.

In the present study, such developmental defects as decreased larval body mass and overall failure to emerge as adults were observed in *O. bicornis* exposed to all chlorpyrifos and cypermethrin treatments. The efficiency of larvae in converting pollen into wet body mass was considerably hampered by Dursban-chlorpyrifos and Sherpa-cypermethrin, producing smaller larvae than the control ones. Although Mospilan-acetamiprid did not provoke a decrease in larval survival or body mass, it accelerated cocoon spinning initiation. Such an effect can be explained by altered brain function due to neonicotinoids mode of action by disrupting nerve signals through binding permanently to the nicotinic receptors (Shi et al., 2019). In support of this,

Jacob et al. (2019) observed that the acetamiprid in Mospilan led to motor dysfunction in orally fed stingless bees, *Scaptotrigona postica*, by disrupting neuromuscular systems.

Many studies have shown that the observed insecticidal toxic effects such as developmental delay, altered diapause behaviour, decreased immunity, etc., during bee larval development could be caused by the alterations at the cellular and molecular level (Tomé et al., 2020). Further, insecticides usually influence neuro-molecular interactions by altering neurohormonal secretions. For example, α-cypermethrin induced hypo-glycaemic and hypotrehalosaemic responses with decreased ATPase and acetylcholinesterase activities in emerging honey bees (Bendahou et al., 1999). All these insecticide-driven changes at the biochemical level could explain the overall developmental failure of individuals for all tested insecticides as compared to the controls in this study. In turn, the accelerated pupation of the Mospilan-ACT treated larvae may explain their lower winter survival probability because the duration of the prepupal summer dormancy is known to be the main driving factor synchronising the eclosion of adult *Osmia* bees with the onset of wintering temperatures (Sgolastra et al., 2012). Because some pupae/adults might have died inside the cocoons even before overwintering (which was not observed visually in the experiment), the effect of insecticides on winter survival could not be estimated directly. However, *O. bicornis* weight loss during hibernation in cocoons was uniform across treatments, including the controls. It is worth noting that among the emerged bees in almost all pesticide-treated groups, the ratio of females to males was very low (in a few cases no females emerged at all; see Table 2), indicating on possibly higher toxicity of the pesticides to females than to males, as found by Mayack and Boff (2019) and Sandrock et al. (2014). However, as only a few bees emerged in the pesticide treated groups, this supposition has to be treated with caution because the sex ratio is based on very small samples sizes and additionally the initial sex ratio of larvae used in the experiment is unknown.

Organophosphates make up to 40% of all insecticides used worldwide (Hayat et al., 2018). In Europe, chlorpyrifos has been commonly used for numerous crops such as rapeseed, winter wheat and vegetables (Gill et al., 2013), and detected in living and dead honey bee samples (Kiljanek et al., 2017). Recently, the use of chlorpyrifos has been banned in the EU (European Commission, 2020), but it is still used in other parts of the world. As an alternative – due to their broad spectrum of action and relatively low doses of application – pyrethroids are currently used on a large scale, mainly cypermethrin in fruit orchards (Oliver et al., 2015), and hence have also been detected in many bee-related products (Mullin et al., 2010). In parallel, the systemic neonicotinoid, acetamiprid, has been extensively used to control pests in various agricultural crops (Kurwadkar and Evans, 2016) and has also been detected in several bee nests (Jacob et al., 2019; Mullin et al., 2010). Our work has clearly shown that all three tested insecticides may cause unacceptable damages to the developing *O. bicornis* bees even at very low concentrations in pollen, making populations of solitary bees at stake with the current insecticide usage regulations. Among the three plant protection products used in this study, the acetamiprid-based Mospilan appeared to be less lethal to the larvae than the other insecticides tested but the overall developmental success rate was still significantly lower than in control.

The interesting phenomenon observed in the study was the lack of clear monotonic concentration dependency for the studied endpoints. In the case of all three plant protection products a significant increase of larvae mortality and/or decrease in adult emergence rate was observed already at the lowest concentrations and only at the highest Dursban-CHP concentration all larvae died while no significant concentration-response relationship was found for remaining concentrations of all three pesticides. At the moment we can only speculate why this was the case, with one possible explanation being the effective degradation and excretion of the insecticides at higher concentrations – we cannot exclude that degradation rate of insecticides increases with increasing internal concentration. Such a flat response across concentrations spanning over two orders of magnitude certainly deserves further studies, especially that similar phenomenon was found also by other authors. For example, Abbott et al. (2008) found that the time to reach the last larval stage (~30 days) in female *Osmia lignaria* larvae was not dose-dependent when exposed orally by injecting imidacloprid at 0, 3, 30 and 300 ppb into the pollen using Hamilton syringe. Similarly, Anderson and Harmon-Threatt (2019) observed that upon chronic contact exposure with 7.5, 15 and 100 ppb imidacloprid in saline solution, the development speed and the body mass of the solitary bee *Megachile rotundata* were not dose-dependent at different life stages (pre-pupa, pupa and pre-emergent adults). In the same study, the authors also observed that the adult female longevity of *Osmia cornuta* followed an inverted u-shaped pattern with a slight increase in longevity at low concentrations of imidacloprid (i.e. 7.5 and 15 ppb) and a decrease at high concentrations (i.e. 100 ppb) compared to controls (Anderson and Harmon-Threatt, 2019).

Even though there were no significant differences in the measured endpoints between insecticide concentrations in our study (except for the highest Dursban-CHP treatment), clear toxic effects were visible in all insecticidal treatments compared to controls, despite the relatively high mortality of control individuals. The lower survival of controls (43%) could be due to the residues of eight pesticides, including acetamiprid and chlorpyrifos, found in the commercial pollen used in the study, although the concentrations of pesticides in control pollen, even combined with the lowest treatment concentrations, were at least an order of magnitude below the maximum environmental concentrations observed by Mullin et al. (2010). On the other hand, mean larval mortality up to 28.3% and mean pupal mortality up to 27.9%, with the highest overall mortality in both stages caused by factors other than parasitism as high as 47.5% was recorded in the field by Steffan-Dewenter and Schiele (2008). Even if the observed control mortality in our study was caused by the cumulative action of the eight pesticides found in control pollen, the differences between control and insecticide-treated bees are exclusive to the particular insecticidal treatments. Nevertheless, our study emphasises the importance of screening the experimental pollen for pesticide residues, which is not a usual practice in similar studies (Dai et al., 2019; Sgolastra et al., 2015; Abbott et al., 2008; Tesoriero et al., 2003).

The current risk assessment schemes for pesticides in bees follow a tiered approach for toxicity testing: tier 1 – laboratory assays based on acute exposure and $LD_{50}$ estimates, and tier 2 to 3 – semi field and field tests (EFSA, 2012). Unfortunately, the sublethal effects on bees' development to healthy adults when exposed at larval stages are overlooked in tier 1 laboratory testing, although claimed to be detectable in either tiers 2 and/or 3. However, it is highly probable that if an agrochemical exerts no relevant toxicity in tier 1, it will unlikely be tested in the higher tiers. From our study it is evident that even if a pesticide does not cause any toxicity in the early stages of development as larvae, it can still affect the overall development, as seen for Mospilan-ACT in this study. Moreover, sublethal effects at the semi-field and field level are conducted using honey bees, ignoring the impacts on solitary bees (Sgolastra et al., 2020). Therefore, future research ought to focus on developing more sophisticated yet cost-effective protocols for testing lethal and sublethal effects in both the adult and larval life stages of solitary bees, aiming to be implemented in tier 1 risk assessment for pesticide regulations. Recently, Eeraerts et al. (2020) summarized few recommendations for standardized oral toxicity test protocols for larvae of solitary bees, *Osmia spp.*, and suggested to use 1 to 5 concentrations of the test pesticide together with 1 negative and 1 positive toxic control. From our study, it appears necessary to test at least 3 pesticide concentrations, covering approximately 1:1000 range (including a field-realistic concentration) to obtain a reliable concentration-response relationship. It is also evident from our study that the suggested end points - the development to second instar larva, cocoon initiation and adult emergence since treatment at first instar stage of

larvae with laboratory contaminated pollen are useful for testing lethal toxicity (Eeraerts et al., 2020).

## 5. Conclusions

Our study showed that exposure to Dursban-CHP and Sherpa-CYP at environmentally realistic concentrations in pollen decreased survival rate and body mass of *O. bicornis* larvae and, as a consequence, decreased the overall developmental success measured as survival to adulthood. Mospilan-ACT at environmentally realistic concentrations in pollen did not affect larvae survival but shortened time to cocoon formation and significantly decreased the adult emergence rate. These findings contribute to the accumulating body of evidence showing that some plant protection products cause unacceptable developmental perturbations to solitary bees, in our study represented by *O. bicorns*, even at concentrations actually observed in pollen in agricultural fields, indicating an urgent need for revising current pesticide usage recommendations.

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## CRediT authorship contribution statement

**Jaya Sravanthi Mokkapati:** Conceptualization, Methodology, Resources, Formal analysis, Writing – original draft. **Agnieszka J. Bednarska:** Conceptualization, Supervision, Formal analysis, Writing – review & editing. **Ryszard Laskowski:** Conceptualization, Supervision, Formal analysis, Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.145588.

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