Larval oral exposure to thiacloprid: Dose-response toxicity testing in solitary bees, *Osmia* spp. (Hymenoptera: Megachilidae)

Gregor Claus [a,*], Matti Pisman [b], Pieter Spanoghe [a], Guy Smagghe [b], Maxime Eeraerts [b]

[a] *Laboratory of Crop Protection Chemistry, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium*
[b] *Laboratory of Agrozoology, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium*

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ABSTRACT

Risk assessment of pesticides involves ecotoxicological testing. In case pesticide exposure to bees is likely, toxicity tests are performed with honey bees (*Apis mellifera*), with a tiered approach, for which validated and internationally accepted test protocols exist. However, concerns have grown regarding the protection of non-*Apis* bees [bumble bees (*Bombus* spp.), solitary and stingless bees], given their different life cycles and therefore distinct exposure routes. Larvae of solitary bees of the genus *Osmia* feed on unprocessed pollen during development, yet no toxicity test protocol is internationally accepted or validated to assess the impact of pesticide exposure during this stage of their life cycle. Therefore, the purpose of this study is to further validate a test protocol with two solitary bee species (*O. cornuta* and *O. bicornis*) to assess lethal and sublethal effects of pesticide exposure on larval development. Larvae were exposed to thiacloprid (neonicotinoid insecticide) mixed in a new, artificial pollen provision. Both lethal (developmental and winter mortality) and sublethal endpoints (larval development time, pollen provision consumption, cocoon weight, emergence time and adult longevity) were recorded. Effects of lower, more environmentally realistic doses were only reflected in sublethal endpoints. In both bee species, thiacloprid treatment was associated with increased developmental mortality and larval development time, and decreased pollen provision consumption and cocoon weight. The test protocol proved valid and robust and showed that for higher doses of thiacloprid the acute endpoint (larval mortality) is sufficient. In addition, new insights needed to develop a standardized test protocol were acquired, such as testing of a positive control for the first time and selection of male and female individuals at egg level.

1. Introduction

Multiple insect taxa are indispensable for insect-mediated pollination of both wild flowers (Ollerton et al., 2011) and agricultural crops (Eeraerts et al., 2019; Klein et al., 2007). However, in recent decades, worldwide declines of insect populations have been detected in both natural habitats and agricultural landscapes (Seibold et al., 2019; Wagner, 2020). Likewise, these declines have also been detected for key insect pollinators such as wild bees, wasps and hoverflies. Multiple interacting stressors such as changing climatic conditions, habitat loss, invasive species and pathogens are main drivers (Goulson et al., 2015). Widespread pesticide use in agricultural landscapes has resulted in significant contamination of nectar and pollen (Botías et al., 2015). Toxic effects of pesticides towards non-target organisms cannot be excluded,

hence pesticides were added to the list of main drivers of pollinator declines. In particular, the link between pollinator loss and neonicotinoid insecticide use has caused much debate (IPBES, 2016). Indeed, neonicotinoids have been detected in pollen and nectar (Chauzat et al., 2006). Pollinators are therefore chronically exposed to sublethal concentrations, negatively affecting behavior such as foraging and reproduction (Rundlöf et al., 2015).

Neonicotinoids have gained tremendous importance since their introduction in the 1990s: they drove other classes of insecticides such as organophosphates and carbamates out of the market and grew to a total insecticide market share of 24% in 2008 (Jeschke et al., 2011), keeping this position at least until 2015 (Bass et al., 2015). Of this global market share, 86% can be attributed to thiamethoxam, imidacloprid, and clothianidin; thiacloprid accounts for 3.8%. Neonicotinoids are

agonists of the nicotinic acetylcholine receptors (nAChRs), and possess high specificity for insect over vertebrate nAChRs, hence are highly toxic to insects but less to humans (Liu et al., 1993). Neonicotinoids are systemic, thus taken up by and transported to all plant parts through the xylem, offering crop protection against plant tissue piercing and sap-sucking insects, such as aphids (Magalhaes et al., 2009). Neonicotinoid use has been rigorously reviewed by the European Food Safety Authority (EFSA) and as a consequence was highly restricted in the European Union (EU). Neonicotinoid seed coatings have been banned in the EU unless used in permanent greenhouses. Only two neonicotinoid active substances remained on the European market which can be used only under strict limitations of spray applications on flowering crops and weeds. However, across the globe varying regulations regarding neonicotinoids exist and seed coatings are still being used to a considerable extent in, for example, the United States, Canada and Australia (see Supplementary Material, Section S1).

EFSA proposed a tiered risk assessment of plant protection products and their active substances on honey bees (*Apis mellifera*), bumble bees (*Bombus* spp.) and solitary bees (EFSA, 2013). However, in the screening and first tier risk assessment, lethal endpoints of honey bees can still be used with an assessment factor (AF, safety factor) of 10, to assess the risk for bumble bees and solitary bees. The reason for this is mainly that at the time, no internationally agreed and adopted guidelines existed for bumble bees and solitary bees. This demonstrates that European risk assessment heavily relied on the eusocial honey bee, while most of the bees are solitary (Michener, 2007). Guidelines of the Organisation for Economic Co-operation and Development (OECD) for acute contact and oral honey bee toxicity were available in 1998 (OECD Tests No. 213 and 214), and acute oral and contact bumble bee protocols have been developed in 2017 (OECD Tests No. 246 and 247). For all other situations official test protocols have yet to be developed. Currently, first tier acute oral and contact test protocols for adult solitary bees (*Osmia* spp.) are being developed by the non-*Apis* working group of the International Commission for Plant-Pollinator Relationships, with the aim of establishing an OECD guideline (Roessink et al., 2018). In the United States, risk assessment for pollinators still remains completely honey bee-centered.

Dissimilarities in life history traits between solitary and social bee species make that exposure and sensitivity towards pesticides differ. These differences are linked with a possible increased risk: univoltinism with flight period during time of pesticide application, underground nesting cavities in soil close to the treated crop, contaminated nest-building material such as mud from treated fields, short foraging range combined with a nest location close to treated crop, absence of trophallaxis, oligolectic larval diet on pollen originating from treated crops and low body weight (Brittain and Potts, 2011).

For a long time, it has been discussed that using the honey bee as surrogate organism for all bee species in pesticide risk assessment might be an oversimplification (Morandin et al., 2005). In particular, the representativeness of honey bee sensitivity compared to non-*Apis* bee species (*Bombus* spp., solitary and stingless bees) is debatable. Arena and Sgolastra (2014) showed a 2-million fold sensitivity difference between all bee species regarding lethal endpoints (contact and oral toxicity). Yet, in 94.7% of the cases, non-*Apis* bee species were no more than 10 times more sensitive compared to the honey bee, hence EFSA proposed the AF of 10 (EFSA, 2013). However, this AF is dependent on chemical class: for neonicotinoids an AF of 10 protects only 88.8% of all bee species. Furthermore, the AF should only be applied to acute endpoints, not to chronic endpoints or larval development endpoints. Indeed, no sensitivity differences on acute endpoints between adult honey bees and solitary bees were shown (Uhl et al., 2019). However, the relative sensitivity of solitary bees to honey bees for chronic and developmental endpoints remains unknown.

Especially the lack of a tier 1 larval development test protocol for solitary bees in pesticide risk assessment raises concerns, considering oral exposure of unprocessed pollen is mainly during this life stage

(Eeraerts et al., 2020; Sgolastra et al., 2015). A larval test protocol is suitable to assess, apart from lethal effects, sublethal effects, for example, on development and longevity. Sublethal effects through pesticide exposure during the larval stage were shown for honey bees on larval development and body weight (Wu et al., 2011). Sublethal effects on bumble bee larvae have also been reported (Siviter et al., 2020). However, presently, sublethal effects for bumble bees and solitary bees are not yet taken into consideration in the current risk assessment scheme (EFSA, 2013). Nonetheless, EFSA (2013) is inclined to further integrate *Osmia* spp. in their risk assessment scheme, because they are considered suitable test organisms for several reasons: testability, possibility to rear, well-known biology, economic relevance and representative for aboveground nesting bees. Furthermore, *Osmia* spp. meet the additional criteria of focal species: ecological relevance (pollination), and expected exposure and sensitivity towards pesticides (EFSA Scientific Committee, 2016; Hilbeck et al., 2014; Romeis et al., 2011). To account for inter-species variability, an appropriate AF will have to be used. The absence of official standardized test protocols for solitary bees, both for larval and adult stages, has led to difficulties in comparison and interpretation of the limited available toxicity tests reported in literature (Abbott et al., 2008; Dharampal et al., 2018; Konrad et al., 2008; Nicholls et al., 2017; Sgolastra et al., 2015; Tesoriero et al., 2003). Therefore, this study aims to develop a standardized oral toxicity protocol for solitary bee larvae in the context of screening and first tier evaluation of (active substances of) plant protection products, based on a recent literature review by Eeraerts et al. (2020). This research presents results of this protocol, where larvae of two solitary bee species, *Osmia cornuta* (European orchard bee) and *O. bicornis* (red mason bee), were exposed via their food provision to different doses of thiacloprid, in order to identify relevant endpoints for both lethal and sublethal effects. In addition, certain indispensable technical aspects for a standardized toxicity protocol, such as the inclusion of a positive toxic control and the selection of female bees on the basis of the egg stage, were validated.

2. Materials and methods

2.1. Description of study organisms

O. cornuta and *O. bicornis* are univoltine, solitary bee species that nidify in existent cavities. In March−April, the bees emerge from their cocoons; first males, who had occupied the outer brood cells of the nesting cavities, followed by females who had occupied the inner brood cells. After mating, female bees collect pollen and nectar (hereafter: "pollen provision") for provisioning brood cells. Eggs are laid on a pollen provision and a mud wall is built as partitioning between cells (Fabre, 1915). *Osmia* spp. eggs enclose first larval instars, which only feed on embryonic fluids. After ecdysis, second larval instars start feeding. Larvae go through another three moults, while continuing feeding, and ultimately reaching the fifth larval instar, which defecates for the first time, and is then called a prepupa. When the pollen provision is completely consumed, prepupae initiate cocoon spinning. Pupation occurs inside the cocoon and adult bees emerge from the pupae in September and overwinter in the cocoon in a diapausal state (Raw, 1972).

2.2. Bee populations and rearing method

Bees (overwintering adults in cocoons) were obtained from Inbuzz Extra (Laren, The Netherlands) and were stored at 4 °C before the start of the experiment. Four wooden laminar trap nests, supplied by Moors Tuinen (Maasbree, The Netherlands), were set up in the ecologically managed garden (no use of pesticides) of the Faculty of Bioscience Engineering, with the nest entrances facing south. Pollinator-attractive plants were available [black hellebore (*Helleborus niger*), apple (*Malus domestica*), grape hyacinth (*Muscari botryoides*), apricot (*Prunus armeniaca*), sweet cherry (*Prunus avium*), blackthorn (*Prunus spinosa*), pear

(*Pyrus communis*) and goat willow (*Salix caprea*)] to ensure sufficient food availability from March to May. Trap nests consisted of 21 wooden grooved boards (18.0 cm long, 16.0 cm wide, 1.4 cm high) with 12 U-shaped nesting cavities (8 mm wide), separated by PVC sheets (0.18 mm thick), allowing for daily inspection of newly laid eggs without disturbance (Fig. S1). Nests were placed outside on 14 March 2019, together with 500 cocoons of *O. cornuta* in Tetra Pak® boxes to protect them from light and rain. One week later, 500 cocoons of *O. bicornis* were placed outside. Nests were set up 1 m above ground and were sheltered from water and birds. In April 2019, when female bees had emerged, trap nests were opened and every wooden board was visually inspected three times a week to check whether egg laying had started.

2.3. Thiacloprid treatments

Technical grade thiacloprid with a purity of 98.9% was obtained from Bayer CropScience (Leverkusen, Germany). A thiacloprid stock solution of 1 500 mg/kg was prepared by adding 25 mg of thiacloprid to 50 g of a sucrose solution (sugar + water,1:1 m/m). Pollen provisions were prepared freshly prior the test, by mixing honey bee pollen obtained from Biobest® (Westerlo, Belgium) and sugar water (9:1 m/m). Test solutions were obtained from the stock solution by dilution with sugar water (4:1; 24:1; 249:1 and 2 499:1 m/m) and were stored at 4 °C during test performance. All larvae received a new pollen provision of 350 mg (fresh weight) (Eeraerts et al., 2020). This resulted in five thiacloprid treatments: 0.020 mg/kg (T1, 0.007 µg/bee); 0.20 mg/kg (T2, 0.070 µg/bee); 2.0 mg/kg (T3, 0.700 µg/bee); 10 mg/kg (T4, 3.50 µg/bee); 50 mg/kg (T5, 17.5 µg/bee). The lower three thiacloprid treatments were comparable to actual field concentrations [own unpublished data; Botías et al. (2015)] and were supplemented with two high doses to facilitate a dose-response relationship. This test with five increasing pesticide concentrations can be considered as a range-finding test, with concentration ratios not exceeding a factor of 10. Negative (T0, blank sugar water, 0 mg/kg) and positive control treatments with dimethoate (T6, 35.0 mg/kg or 12.3 µg/bee) were included in the trial. Dimethoate (Perfekthion® 400 EC) was obtained from BASF (Waterloo, Belgium). Treatments were placed in separate 48-well sterile crystal-grade polystyrene culture plates with an internal diameter of 9.75 mm and a depth of 1.75 cm (SPL Life Sciences, Pocheon-si, South Korea). Blank and treated pollen provision samples were analyzed for dimethoate and thiacloprid residues (Supplementary Material, Section S3).

2.4. Incubation and monitoring

Grooved boards containing eggs were taken out the trap nests and were brought to the laboratory where they were kept at room temperature (±20 °C). Eggs were transferred from the nest to their respective new pollen provision with a micro spatula and were randomly assigned to treatments. Egg collection happened throughout the natural egg laying period which was for *O. cornuta* between 4 and 26 April and for *O. bicornis* between 26 April and 9 May. Because the trap nests were monitored on a daily basis, freshly laid eggs could be selected (L1 stage). The two *Osmia* species could be readily distinguished based on original pollen provision appearance. Pollen provisions of *O. cornuta* are homogenously humid, whereas those of *O. bicornis* are covered with dry pollen. The position of the brood cell in the nesting cavity of each egg that was harvested was noted in order to retrospectively link the sex of each hatched bee to its position in the nesting cavity. A minimum sample size of 30 larvae per treatment group was targeted. Subsequently, eggs were incubated in darkness at 22 ± 1 °C and 60 ± 3% relative humidity in a PHCbi MLR-325H climate chamber (Koizumimachi, Japan). Eggs failing to reach second larval instar were discarded as we considered these damaged by the transfer (i.e. eggs were visibly punctured, see Fig. S.2). Larval development was monitored three times per week until cocoon spinning. The dates of the start of the second larval instar, larval

mortality and the start of cocoon spinning were recorded. Prewinter incubation was initiated on 9 September 2019, when pupae had completed metamorphosis within the cocoon into adults, by lowering temperature to 14 °C for a period of 2 weeks (Bosch and Kemp, 2004; Eeraerts et al., 2020). Preceding transfer to round Petri dishes (55 mm diameter, Corning® Gosselin™, product number: BP53–06), cocoons (with adult bees inside) were weighed accurately to 0.001 g, as well as the pollen provision remainder, with an analytical balance (Sartorius LA620P, Göttingen, Germany). Subsequently, cocoons were subjected to a wintering period of 105 days at 4 °C (Bosch and Kemp, 2004; Eeraerts et al., 2020). On 6 January 2020, cocoons were incubated in the dark at 24 ± 1 °C and 60 ± 5% relative humidity. Sex and species were confirmed upon bee emergence by monitoring on a daily basis. Bees were deprived of food and date of mortality following starvation was recorded. Daily monitoring was prolonged until 12 April 2020, leaving the bees ample time to emerge from their cocoons within an ecological relevant time frame.

2.5. Statistical data analysis

Statistical analyses were performed in R version 3.5.1 (R Core Team, 2018) using RStudio version 1.1.463. With one-proportion Z tests the predictions of bee species and sex were verified. Effects of thiacloprid and dimethoate on mortality, larval development and feeding and adult fitness of *O. cornuta* and *O. bicornis* were evaluated. This was done by comparing differences between treatment groups in larval mortality (mortality before cocoon initiation or developmental mortality rate), adult emergence (mortality after cocoon formation or winter mortality rate), larval development time (time from second larval instar up to cocoon initiation), pollen provision consumption, cocoon weight, emergence time and adult longevity. Larval mortality and adult emergence were analyzed using generalized linear models (GLMs) with binomial distributions and the logit link function. Mortality was scored as a binary variable (with 1 being dead and 0 being alive). For cocoon weight data, analysis of variance (ANOVA) model assumptions were verified with the Shapiro-Wilk normality test and Levene's test for homogeneity of variance (function "*leveneTest*" in "*car*" package version 3.0.2)], besides a graphical exploration with QQ plots of ANOVA model residuals and box plots. Tukey's 'Honest Significant Difference' method was used to produce adjusted *p*-values, to account for multiple comparisons. Larval development time, emergence time and adult longevity (all count data) were analyzed with GLMs but with Poisson distributions and the log link function. In case of overdispersed count data, Conway-Maxwell-Poisson distributions were used instead with the package "*glmmTMB*" version 1.0.1. The impact of the actual consumed thiacloprid dose on larval development time, cocoon weight, emergence time and adult longevity was assessed with linear regression. Visualization of GLMs was performed with packages "*emmeans*" version 1.4.7, "*effects*" version 4.1.4 and "*ggplot2*" version 3.3.2. Finally, a correlation analysis (Pearson's product-moment correlation) was performed on the effects of thiacloprid. Assumptions were visually inspected (linearity of correlation and normal distribution of effects). Research results are reported with a 95% confidence interval.

3. Results and discussion

3.1. Lethal endpoints

3.1.1. Larval development mortality rate
Information regarding the pesticide residue analysis of pollen provisions can be found in the Supplementary Material, Section S3 (Tables S1–S3). In this study 327 *O. cornuta* and 544 *O. bicornis* larvae were sampled (Table S4). Larval development mortality rate was not statistically different between species, comparing each treatment group pairwise (Fig. 1 and Table S5). Negative control mortality rate (T0) was on average 4.7% and 22.2% for *O. cornuta* and *O. bicornis* respectively.
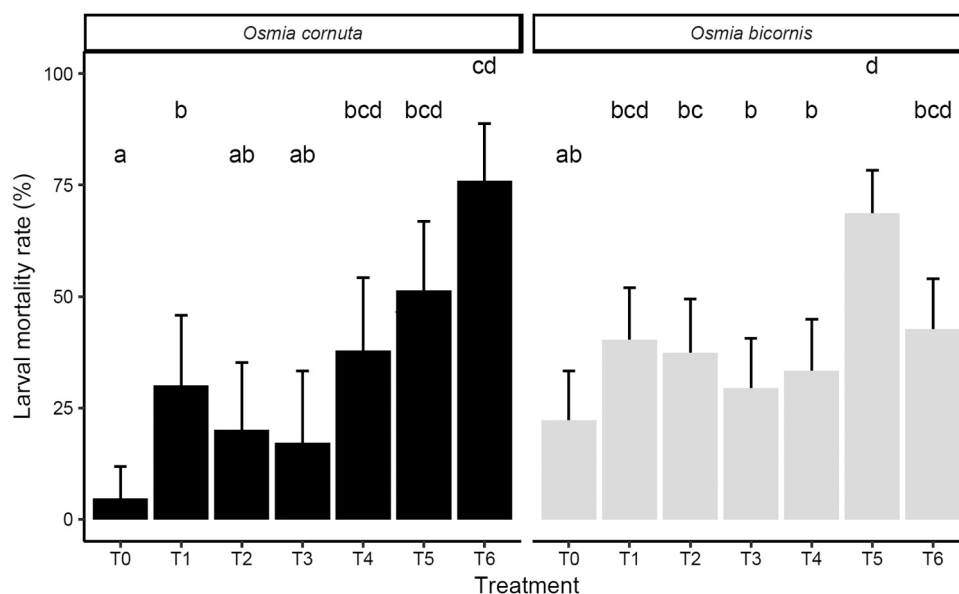
Fig. 1. Bar chart of the mean larval mortality rate of *O. cornuta* ($n = 327$) and *O. bicornis* ($n = 544$) due to pesticide pollen provision treatment. Error bars indicate 95% confidence intervals. Different letters indicate significant statistical differences for pairwise comparisons (Tukey's contrasts, $\alpha = 0.05$).

Other researchers reported similar developmental mortality rates in negative control groups, ranging from 6.5% to 20% (Bosch and Kemp, 2001; Konrad et al., 2008; Nicholls et al., 2017; Sgolastra et al., 2015; Tesoriero et al., 2003).

For *O. cornuta*, the developmental mortality rate in T2-T3 was not different compared to T0. However, T1 and T4 revealed an increased developmental mortality rate compared to T0. For *O. bicornis*, in all the lower thiacloprid treatments (T1-T4), developmental mortality rate was not different compared to T0.

Tesoriero et al. (2003) applied pesticides at field rate (1 μl) in pollen provisions and found the highest developmental mortality rate in a bio-insecticide treatment (*Quassia amara* extract: 82.8%), followed by a copper-based fungicide treatment (copper oxychloride: 44.8%) and no difference with the negative control group in a strobilurin fungicide treatment (kresoxim-methyl: 13.2%). Konrad et al. (2008) studied the effect of insecticidal proteins, yet no treatment effects on developmental mortality rate appeared. Treated pollen provisions with imidacloprid and clothianidin up to 300 μg/kg were reported to have no lethal effects on *Megachile rotundata* (alfalfa leafcutting bee) and *O. lignaria* (blue orchard bee) (Abbott et al., 2008). In an assay with *M. rotundata* larvae feeding on pollen provisions contaminated with deltamethrin (pyrethroid insecticide) and spinetoram (spinosyn insecticide) at 50–200 μg/kg (realistic field residue levels), no larvae reached the cocoon spinning stage, but treatment with flubendiamide (ryanoid insecticide) did not lead to an increased developmental mortality rate (about 10%) compared to the negative control group (Gradish et al., 2012). Also no effect on developmental mortality rate occurred for clothianidin treatments (up to 10 μg/kg) (Nicholls et al., 2017).

3.1.2. Winter mortality rate

There was no significant effect of pesticide treatment on winter mortality rate (Figs. S3 and S4 and Table S6). The average winter mortality rate was $6.5 \pm 2.1\%$. On the one hand, Sgolastra et al. (2015) found that lethal effects occurred mainly during larval development, not during overwintering. On the other hand, Nicholls et al. (2017) experienced an inexplicably high winter mortality in the negative control group (13.8%), not in the pesticide treatment group (6.2%). A small sample size (31 eggs) and inherent high natural variability of mortality rate [see for example Konrad et al. (2008)] lay at the basis of this seemingly higher winter mortality rate. Konrad et al. (2008) found no treatment effects on winter mortality rate (on average 10.1%). Lastly,

bee larvae offered spirotetramat-treated (tetramic acid insecticide, 1.4 mg/kg) pollen provisions did not show an increased total mortality rate (developmental and winter mortality combined, 22.7% and 16.7% for males and females respectively) (Sgolastra et al., 2015). All these results together suggest that it is relevant to separately report developmental and winter mortality rate.

3.2. Sublethal endpoints

3.2.1. Larval development time

For all bee species and sexes, the highest thiacloprid treatment (T5) resulted in an increased larval development time (Fig. 2 and Table S4). Larval development time was not statistically different between species and sexes, comparing groups pairwise. *O. cornuta* females and *O. bicornis* males had already an increased development time in T4 compared to T0. Linear regression [Fig. S5, determination coefficient (R^2) = 0.391; $p < 0.001$] confirmed that larval development time increased for all bee species and sexes with increasing thiacloprid consumption, confirming a dose-response relationship. *O. cornuta* was less sensitive compared to *O. bicornis* [slopes are respectively: 0.770 ± 0.240 and 1.83 ± 0.28 days/(μg/bee)].

Bosch and Kemp (2001) studied the duration of several developmental stages of *O. lignaria* under differing temperature conditions and discovered that the egg stage duration varies between 6 and 9 days, the duration from first to fourth larval instar ranges from 3 to 9 days, the fifth larval instar lasts 8–13 days and cocoon initiation starts 2–9 days later. The total duration range is 19–40 days with an average of 26 days at 22 °C and 25 days under orchard conditions. In a study with *O. bicornis* very similar results were reported: an egg stage duration of 7.6 days and a feeding stage duration of 31.6 days for males and 36.5 days for females in field conditions (Giejdasz and Wilkaniec, 2002).

Konrad et al. (2008) found only for one of the tested insecticidal proteins [0.1% of *Galanthus nivalis* (common snowdrop) agglutinin] an effect on larval development time (range: 16–35 days) which was significantly longer compared to the negative control group (15–25 days). Other studies have shown no or minor effects of pesticide treatments on larval development time (Abbott et al., 2008; Dharampal et al., 2018; Nicholls et al., 2017; Sgolastra et al., 2015), with a larval development time ranging between 21 and 42 days. For example, Dharampal et al. (2018) did not find an effect of a field-realistic dose of the fungicide propiconazole in the pollen provision (361 μg/kg) on the larval
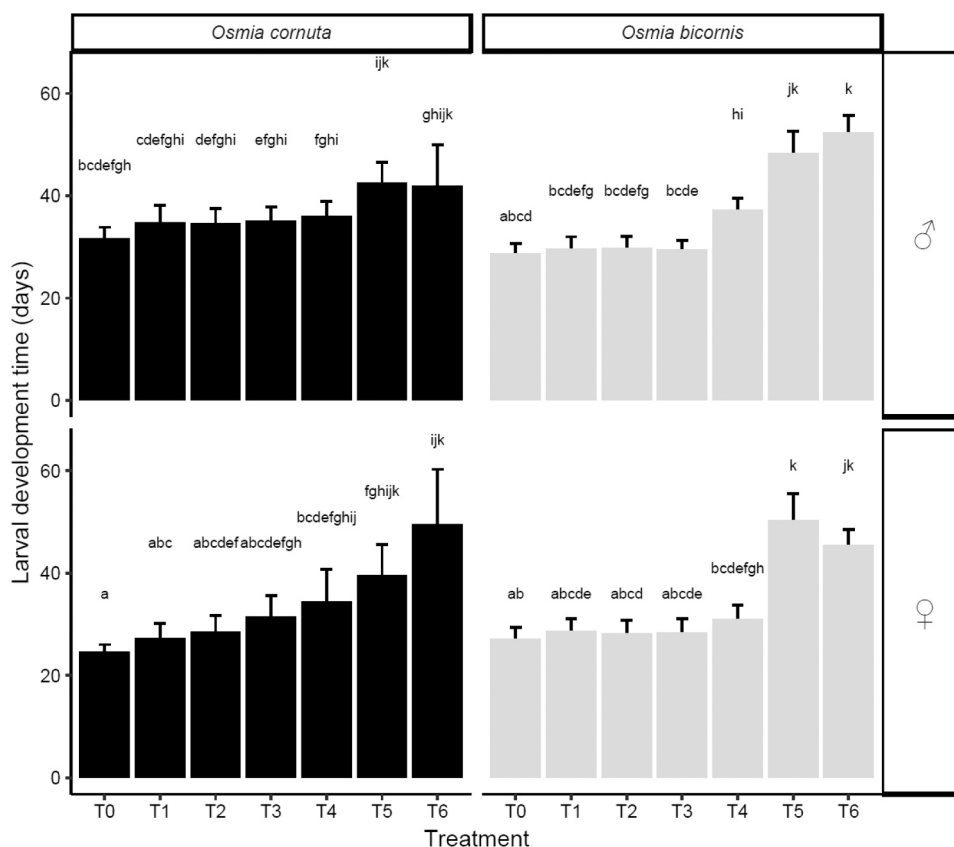
Fig. 2. Bar chart of the mean larval development time of *O. cornuta* (*n* = 212) and *O. bicornis* (*n* = 312) due to pesticide pollen provision treatment. Error bars indicate 95% confidence intervals. Different letters indicate significant statistical differences for pairwise comparisons (Tukey's contrasts, α = 0.05).

development time of *O. ribifloris* (currant mason bee): 28.1 days compared to 25.4 days in the negative control group.

It is not certain whether small differences in larval development time (several days) can have a severe impact on the bee population. However, it has been discussed that it could negatively influence diapause timing (Abbott et al., 2008). Furthermore, larval development time has been negatively correlated with food conversion (pollen provision consumed/body weight), resulting in adults with a reduced body weight (Konrad et al., 2008). Although, effects on food conversion were not observed in previous studies with neonicotinoid treated larvae (Abbott et al., 2008; Nicholls et al., 2017) nor with spirotetramat treated larvae (Sgolastra et al., 2015). Additionally, higher bee fitness and survival has been linked with higher body weight (Bosch and Kemp, 2004). This underlines the importance of larval development time as a sublethal endpoint. Anderson and Harmon-Threatt (2019) attribute a longer development time in pesticide treated larvae to an increased detoxification metabolism, which requires energy and nutrients, lost for normal growth. The idea of an optimum larval development time means that there is a trade-off between developing too fast and too slow. When developing faster, more energy is spent during prewinter as adult. When developing slower, the (pre)pupa has difficulties reaching the adult stage before winter, and (pre)pupae of *Osmia* spp. are unviable to hibernate. In addition, delayed development time implies prolonged exposure to pathogenic infections, parasitism and possible adverse climatic conditions.

3.2.2. Pollen provision consumption

For both species, pollen provision consumption is lower than 350 mg/bee in T4-T5 (Fig. 3, panel A and Table S8). In T0-T3, the amount not consumed is negligible. In T1−T3 *O. cornuta* consumed everything as opposed to *O. bicornis*, who left a very small, insignificant fraction untouched. This implies that pesticide exposure in T0−T3 is

fully according the treatment protocol. In T4-T5 a significant smaller fraction of the pollen provision is taken up, hence a smaller fraction of the pesticide dose is taken up (up to a minimum of 40 mg in T5). Therefore, the actual pesticide dose was calculated per bee in all treatment groups (T0-T6) using the individual consumption data. It is known that *Osmia* spp. under natural conditions start with cocoon spinning as soon as the pollen provision is (almost) completely consumed (Abbott et al., 2008; Bosch and Kemp, 2001; Nicholls et al., 2017). In other toxicity assays with pesticide-treated pollen provisions, bee larvae have fully consumed the provided amount (Konrad et al., 2008; Sgolastra et al., 2015).

It is evident that incomplete pollen provision consumption is linked with a lower adult body weight and size and vice versa (Nicholls et al., 2017; Wilkaniec et al., 2004). This implies that bee larvae exposed to pesticide residues in the pollen provision are potentially harmed on two fronts, both impeding body growth: (1) a reduced consumption and (2) a reduced food conversion (see Section 3.2.1). Higher body weight in female bees has been linked to increased adult longevity and higher ability to reproduce, in terms of creating more brood cells, with a higher proportion of female offspring, and providing a higher quantity of pollen per brood cell (Seidelmann, 2006). This means that there are possibly adverse effects on the *progeny* of larvae initially exposed to pesticides. In other words, it is likely that unwanted detrimental effects of pesticides drag on for at least *two generations* in *Osmia* spp.

3.2.3. Cocoon weight

Cocoon weight was lower in T4 and T5 compared to T0 for all bee species and sexes (Fig. 3, panel B and Table S9). On the one hand, *O. cornuta* female cocoons were heavier than male cocoons up to T4, in T5 this difference leveled out. On the other hand, a sex differentiation in cocoon weight for *O. bicornis* was not observed. Not surprisingly perhaps, as male and female larvae received an equal pollen provision
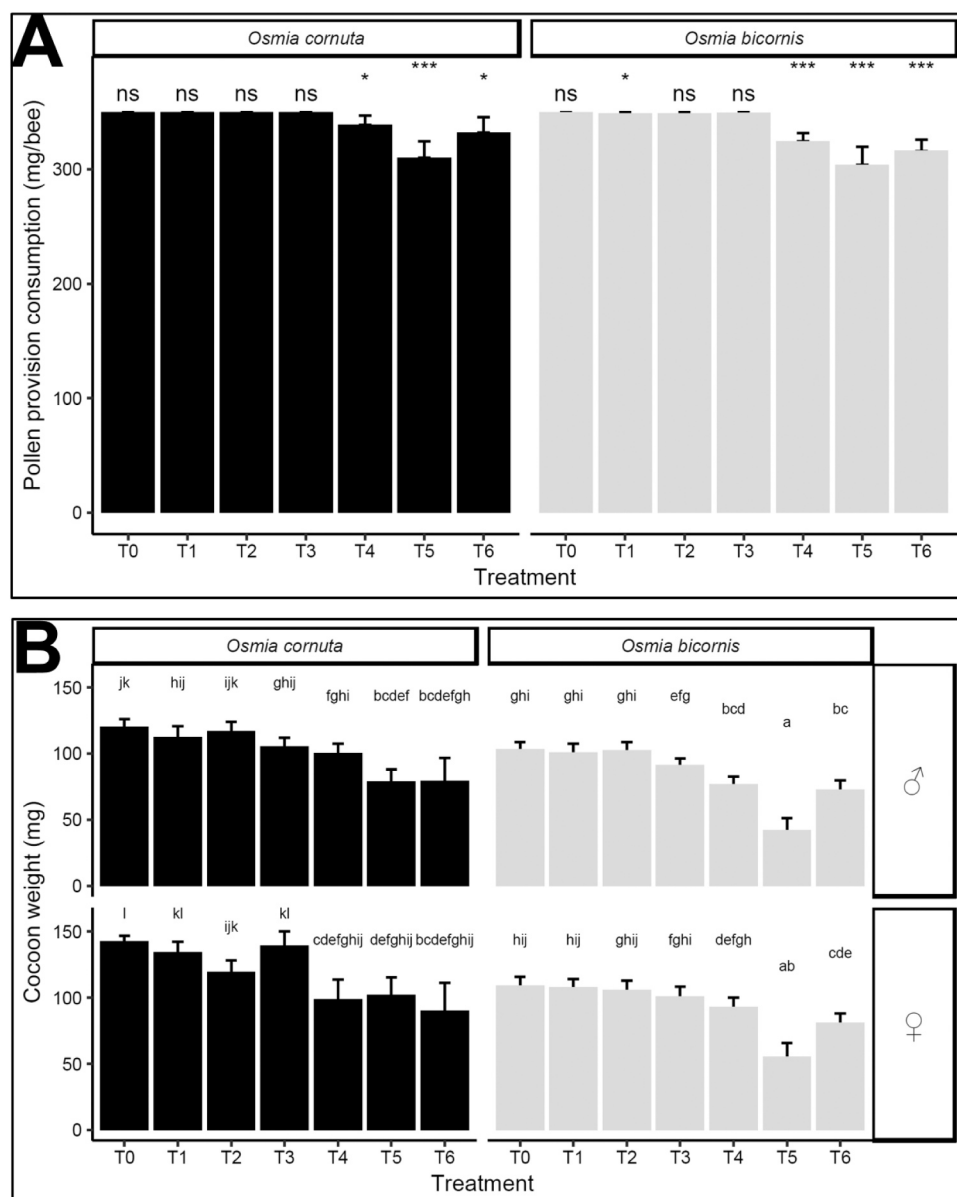
Fig. 3. Panel A: bar chart of mean pollen provision consumption of surviving *O. cornuta* ($n = 212$) and *O. bicornis* ($n = 312$) in the different pesticide treatments. Consumption is compared with complete pollen provision of 350 mg/bee. Indication of significance of *p*-values: ns: $p > 0.05$; *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$. Panel B: bar chart of mean cocoon (+ adult bee inside) weight immediately prior wintering. Different letters indicate significant statistical differences for pairwise comparisons (Tukey's contrasts, $\alpha = 0.05$). Error bars indicate 95% confidence intervals.

mass, which is positively correlated with adult bee and cocoon weight (Wilkaniec et al., 2004). Linear regression (Fig. S6, $R^2 = 0.592$; $p < 0.001$) showed that cocoon weight decreased for all bee species and sexes with increasing thiacloprid consumption, confirming a dose-response relationship. *O. cornuta* bees were less sensitive [slopes are for male: -3.06 ± 0.76 and female: -2.09 ± 0.83 mg cocoon/(µg/bee)] compared to *O. bicornis* bees [male: -5.29 ± 0.88 and female: -4.32 ± 0.86 mg cocoon/(µg/bee)]. Abbott et al. (2008) did not find a dose-response relationship with adult body weight (measured directly after adult emergence) due to imidacloprid exposure, for both *M. rotundata* and *O. lignaria*. Nicholls et al. (2017) encountered similar results with *O. bicornis*: no treatment effect of clothianidin on adult body weight; yet the natural sexual dimorphism in body weight was recorded. Under natural conditions the weight range of female *O. bicornis* cocoons is 70–160 mg (average: 120 mg) and the range for male bees is 50–120 mg (average: 80 mg) (Ivanov, 2006).

The larval stage is of utmost importance for *Osmia* spp. as lipid accumulation in the fat body during this stage is paramount for not only adult *survival* but also adult *vigor*. In this study, cocoon weight is a measure for adult bee vigor. During the (pre)winter stage, when cocooned adult bees are unable to feed, lipids are being mobilized and used as energy source in the midgut. It has been shown that prolonged prewinter stages and warm winter stages result in an increased lipid metabolism, leading to increased body weight loss and winter mortality rate (Torchio et al., 1987).

3.2.4. Emergence time

O. cornuta emergence started from the 6th day of incubation at 22 °C and all bees emerged from their cocoon within 28 days (average: 12.8 ± 0.6 days). By contrast, *O. bicornis* needed about two weeks longer (25.6 ± 1.8 days) and only started emerging from the 13th day of incubation. There were daily new *O. bicornis* emergences up to 54 days after incubation. Eleven *O. bicornis* individuals emerged even later, up to 85 days after incubation (Fig. S7). For all species and sexes there was no effect of pesticide treatment on emergence time (Table S10). However, there seems a drop in emergence time in T5 with female *O. bicornis*. Linear regression ($R^2 = 0.528$; $p < 0.001$) confirmed this negative trend with increasing thiacloprid consumption [slope: -0.684 ± 0.295 days/ (µg/bee)]. Other bees were not sensitive to thiacloprid in terms of emergence time [slope for male *O. cornuta*: -0.049 ± 0.359; female

O. cornuta: -0.107 ± 0.401 and male *O. bicornis*: 0.321 ± 0.331 days/ (µg/bee)].

Timing of emergence can be important for the fitness of bees with regard to the mismatch with flower resources and mating opportunities. Emergence time depends on the duration and temperature of the wintering period and the incubation temperature afterwards (Bosch and Kemp, 2001). Pesticide treatment effects on emergence time are often not found (Abbott et al., 2008; Nicholls et al., 2017; Sgolastra et al., 2015). Only the well-known difference in emergence time between sexes is sometimes apparent: ranging from 0 to 14 days (average: 6 days) for male bees and ranging from 6 to 26 days (average: 16 days) for female bees (Nicholls et al., 2017). This sexual dimorphism is called 'reversed' emergence (Ivanov, 2006). Also Gradish et al. (2012) did not find any pesticide treatment effect on emergence time.

3.2.5. Adult longevity

Longevity in the absence of food was studied for bees that hatched within the time criterion of adult emergence (54 days after incubation). Few differences in longevity were observed within the species-sex groups (Table S11). Male bees of both species lived longer compared to their female counterparts in T0−T2, in T3−T5 this difference was leveled out. Comparing treatments pairwise, *O. cornuta* bees lived equally long as *O. bicornis* bees, both males and females. The ambiguous sensitivity towards longevity was confirmed with linear regression ($R^2 = 0.153$; $p < 0.001$). While female *O. cornuta* and male *O. bicornis* appeared insensitive [slopes: -0.026 ± 0.174 and 0.023 ± 0.157 days/ (µg/bee) respectively], male *O. cornuta* showed a slight negative sensitivity [slope: -0.278 ± 0.166 days/(µg/bee)] and female *O. bicornis* a slight positive sensitivity [slope: 0.275 ± 0.143 days/(µg/bee)].

On the one hand, Konrad et al. (2008) did not find a difference in male or female adult longevity – ranging from 2 to 14 days (average: 7 days) – nor a treatment effect. On the other hand, Sgolastra et al. (2015) found a difference between sexes (difference: male bees lived 1 day longer) ánd a negative treatment effect (treated bees lived 1 day shorter).

Adult longevity, a measure for the bee's vigor, is an important sublethal endpoint, since it reflects the bee's energy reserve: if lipid reserves are depleted during winter due to pesticide detoxification, increased winter mortality rate and decreased vigor occur. Decreased vigor in turn translates into reduced pollen provisioning and is directly linked with a lowered fecundity. Small female adults have a reduced longevity and, above all, are less likely to establish at a nesting site longevity (Bosch and Kemp, 2004).

3.3. Correlation of endpoints

Both assumptions of linear correlation analysis (linearity and normal distribution of data) were verified and proved valid. On the one hand, the correlation between larval mortality rate and development time due to thiacloprid effects was positive and significant ($p < 0.001$, $r = 0.816$, $t = 4.463$, $df = 10$). On the other hand, the correlation between larval mortality rate and pollen provision consumption as well as cocoon weight was negative and significant ($p < 0.01$, $r = -0.798$, $t = -4.181$, $df = 10$ and $p < 0.001$, $r = -0.883$, $t = -5.954$, $df = 10$, respectively). A non-significant correlation was established between larval mortality

rate and effects in a later, adult stage (winter mortality rate, emergence time and adult longevity, see Table 1). This indicates that the majority of effects occur in the larval stage, when the bees are in their most susceptible stage. Furthermore, this evidences that larval developmental mortality rate is more sensitive compared to endpoints in the later developmental stages, and thus suitable as a first tier endpoint. Above all, restricting the toxicity test to this observation (up to June, test duration of 4 months) would substantially reduce the toxicity test time span (compared to higher tier observations, up to March the next year, test duration of 1 year).

3.4. Towards a standardized test protocol

3.4.1. Species and sex prediction

Species identification based on pollen provision appearance proved more successful than the random 50% probability (508/513 or 99% success, $p < 0.001$). Larvae sex could be determined based on brood cell position: larvae in the first brood cell from the back were in more than 75% of the cases female ($p < 0.001$). From the sixth brood cell on, the proportion of female larvae dropped below 30% and no female larvae occurred from the eighth brood cell onwards (Table S12). As the focus is on female workers and queens in the OECD honey bee and bumble bee toxicity protocols, these characteristics are useful for accurate identification of female individuals for future solitary bee larvae toxicity testing.

3.4.2. Positive control group (T6)

An overview of lethal and sublethal effects in both the negative control group (T0) and positive toxic control group (T6) for this oral larval toxicity protocol for solitary bees is provided in Table 2. The positive control was performed with the toxic reference substance dimethoate (12.3 µg/bee), similar to what is used in the OECD honey bee larval toxicity test protocol (8.8 µg/bee, OECD Test No. 237). This treatment serves as a check whether the experimental conditions were valid, in other words, that bee larvae responded to pesticide exposure in general. In this study, the developmental mortality rate for *O. cornuta* in T6 was significantly higher compared to T0, although not for *O. bicornis*. Using Abbott's formula for adjusted mortality (Abbott, 1925), and comparing this to the validity criterion (positive control mortality rate ≥ 50%), a similar inference is drawn: a 75% and 26% developmental mortality rate for *O. cornuta* and *O. bicornis* respectively. Note that treatment groups with the highest thiacloprid dose (T5) had similar mortality rates (51.4% and 68.6% respectively) compared to T6 for both species.

Sgolastra et al. (2015) discussed that a higher negative control mortality rate in laboratory compared to natural conditions arose from the physical manipulation of bee larvae. Hence, they proposed a negative control mortality rate criterion of ≤ 15%, identical to the honey bee larval toxicity test.

Larval development in T6 took significantly longer across both species and sexes. Giejdasz and Wilkaniec (2002) showed that larval development speed increases with increasing temperature and was twice as fast at 28 °C compared to 22 °C. This demonstrates the importance of a constant temperature control during the test protocol. Upon reaching adulthood, a prewinter stage of 2–4 weeks with two or three cooling

Table 1
Correlation matrix (Pearson's product-moment correlation) of thiacloprid effects. Bee species (*Osmia cornuta* and *O. bicornis*) and sex are pooled.

	Larval mortality rate	Winter mortality rate	Larval development time	Pollen provision consumption	Cocoon weight	Emergence time
Winter mortality rate	0.56					
Larval development time	0.82**	0.37ns				
Pollen provision consumption	−0.80**	−0.47ns	−0.93***			
Cocoon weight	−0.88***	−0.49ns	−0.83***	0.84***		
Emergence time	0.10ns	0.24ns	−0.27ns	0.14ns	−0.27ns	
Adult longevity	0.08ns	−0.03ns	0.12ns	−0.08ns	−0.40ns	0.38ns

ns, *, **, ***: indication of significance of *p*-values: ns: $p \geq 0.1$;.: $p < 0.1$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Table 2
Overview of lethal and sublethal endpoints of the positive control group (T6, pollen provision treated with dimethoate, 35 mg/kg) and the negative control group (T0, blank pollen provision) for *Osmia cornuta* and *O. bicornis*. Average values with 95% confidence interval between parentheses are provided.

Endpoints		*O. cornuta*				*O. bicornis*			
		T0		T6		T0		T6	
		♂	♀	♂	♀	♂	♀	♂	♀
<u>Lethal endpoints</u>	Unit								
Larval development mortality rate	(%)	4.7 (1.8–11.9)		76.0 (55.8–88.8)		22.2 (14.1–33.2)		42.7 (32.0–54.0)	
Winter mortality rate	(%)	0 (/)	0 (/)	0 (/)	0 (/)	8.8 (2.9–24.0)	0 (/)	10.5 (2.6–33.7)	0 (/)
<u>Sublethal endpoints</u>									
Larval development time	(days)	31.7 (29.6–33.9)	24.6 (23.3–26.0)	42.0 (35.3–50.0)	49.5 (40.6–60.3)	28.7 (27.0–30.6)	27.1 (24.9–29.4)	52.3 (49.2–55.7)	45.4 (42.5–48.6)
Pollen provision consumption	(mg)	350 (/)		332 (316–348)		350 (/)		316 (305–328)	
Cocoon weight	(mg)	120 (115–126)	143 (139–147)	79 (62–97)	90 (69–111)	104 (98–109)	109 (103–116)	73 (66–80)	81 (74–88)
Emergence time	(days)	12.3 (10.4–14.4)	15.6 (13.8–17.7)	13.4 (10.6–17.0)	11.6 (9.3–14.6)	22.4 (19.5–25.7)	36.8 (32.2–42.1)	24.5 (20.1–30.0)	27.4 (22.8–33.0)
Adult longevity	(days)	9.6 (8.4–10.9)	6.5 (5.8–7.3)	3.4 (2.0–5.9)	3.8 (2.2–6.6)	9.8 (8.6–11.1)	6.6 (5.7–7.7)	10.4 (8.7–12.4)	11.5 (9.9–13.5)

steps are optimal to limit energy demand and at the same time to prevent a temperature shock (Bosch and Kemp, 2001). This test protocol meets this need with a two-week prewinter stage.

Conflicting research results regarding the importance of the pollen source on bee health exist. Wilkaniec et al. (2004) proved that increased pollen quality – pollen mixtures from various plant sources – did not lead to increased body weight; pollen provision quantity was decisive. Contrastingly, earlier work suggested that pollen quality is a major factor to account for in *O. lignaria* (Levin and Haydak, 1957): not one of the honey bee-collected pollen replacements could match the original pollen provision quality, resulting systematically in a reduced body weight. The authors ascribed this to a nutritional deficiency. Lastly, Dharampal et al. (2018) drew attention to a 38.2% reduced prepupal weight and found a significant difference in larval development time between larvae reared on the original pollen provision (20.0 days) compared to larvae fed with artificial pollen provisions made with honey bee-collected pollen (33.2 days). It follows that offering nutritionally uniform and homogenized pollen provisions is essential in larval test protocols. Therefore it is concluded that, with the ambition to make it possible to compare the results from oral larval toxicity tests with solitary bees across different studies, larvae should be offered pollen provisions made with homogenized honey bee-collected pollen instead of with the original pollen provision. Finally, the complete pollen provision was consumed in T0 for both species, indicating that the proposed 350 mg/bee is adequate but not excessive. For both species, consumption in T6 was significantly reduced. Due to this partial consumption, actual dimethoate exposure was slightly (<10%) lower than anticipated.

It was established that overwintering weight loss (25–45%) is not affected by pesticide treatment (Konrad et al., 2008; Nicholls et al., 2017). Although, weight loss has been discussed as a sublethal endpoint (Bosch and Kemp, 2004; Eeraerts et al., 2020) and adverse effects of increased overwintering weight loss on winter mortality and adult longevity were shown for bumble bees (Fauser et al., 2017). Therefore, it is proposed that overwintering weight loss should be included in future oral larval toxicity protocols.

4. Conclusions

This study has proposed an oral larval toxicity protocol for solitary bees and showed its applicability with an example of a relevant neonicotinoid insecticide, thiacloprid. A clear dose-response relationship of larval mortality, i.e. an arrested larval development, due to oral pesticide exposure via the pollen provision of the bee species *Osmia cornuta* and *O. bicornis* was demonstrated. Winter mortality was not implicated

by pesticide treatment, indicating that the majority of *lethal* effects occurred during larval development, which is the most susceptible stage. A higher susceptibility in this larval stage can be explained by the higher exposure expressed on body weight basis. Therefore, developmental mortality rate is the recommended lethal endpoint to take into account when performing the larval oral toxicity risk assessment. More evidence that the larval stage is of utmost importance for the fitness of the solitary bee was shown: larvae exposed to thiacloprid in the diet were prone to a lower pollen provision consumption – although they were spending more time developing – resulting in bees with a reduced body weight and consequently reduced fitness and survival chances. Pollen provision consumption, larval development time and cocoon weight proved to be relevant *sublethal* endpoints with clear distinctions between the positive and negative control group. This study also identified different sensitivities of the two bee species for certain endpoints. Therefore, it seems likely that the responses of other, more diverse bee species will differ, which underlines the importance of including different species in the risk assessment of pesticides. With this study, it has been demonstrated that the oral larval toxicity test protocol for solitary bees is a feasible and relevant tool, ready to be integrated in the routine pesticide risk assessment on bees.

CRediT authorship contribution statement

Gregor Claus: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Matti Pisman**: Conceptualization, Methodology, Validation, Writing - review & editing, Supervision. **Guy Smagghe**: Writing - review & editing, Supervision. **Pieter Spanoghe**: Writing - review & editing, Supervision. **Maxime Eeraerts**: Conceptualization, Methodology, Validation, Writing - review & editing, Supervision.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112143.

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