WildPosh

D2.4 Report of the novel or updated protocols testing the sensitivity of wild insect pollinators to pesticides

19/12/2024

Lead beneficiary: MLU

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1 Preface

This document is a deliverable for the WildPosh project, funded under the European Union's Horizon Europe Research and Innovation Action under grant agreement No. 101135238. The aim of this document is to present the protocols developed by WildPosh partners in WP2 to enable the testing of a range of wild insect pollinator species to pesticides.

2 Summary

An overarching goal of the WildPosh project is to assess the sensitivity of European wild insect pollinators to pesticides, for which novel or updated protocols to maintain those insects in the laboratory are a pre-requisite. This deliverable provides as an appendix the most recent protocols that Partners in WP2 have developed to test wild bee species, wild Lepidoptera (butterfly and moth) species and wild syrphid fly (hoverfly) species to pesticides. Protocols incorporate the topical route of exposure (contact) as well as via ingestion (oral). They also include protocols for exposure in the juvenile (larval) stage as well as the adult stage, and they allow testing of lethal as well as sublethal effects through, for example, chronic exposure.

Protocols have been developed through (i) refinement of existing methodology developed for honey bees, bumble bees and mason bees, (ii) updating of PoshBee protocols for testing wild bee species, and, through a process of trial and error, their refinement by WP2 Partners to additional target wild bee species and other target wild insect pollinator groups (Lepidoptera and syrphid flies). The WildPosh protocols are our current best practice as of 31.12.2024. They will be employed in continued pesticide testing in 2025, whereupon additional adjustments to the protocols are foreseen. As well as permitting ecotoxicological testing of the range of Europe's most important groups of wild insect pollinators to pesticide exposure within Tasks 2.2 and 2.3, these protocols will also form the basis of the improved science-based protocols and model systems (target species groups) for regulatory testing, to be developed in Task 2.4.

3 List of abbreviations

EFSA	European Food Safety Authority
EU	European Union
GA	WildPosh General Assembly
LD50	Lethal Dose killing 50% (of subjects)
M3	Milestone 3
WP2	WildPosh Work Package 2

4 Introduction

Biodiversity is in decline, including that of wild insect pollinators (Potts et al. 2016). The strongest evidence for an EU decline in insect pollinators is for wild bees (Biejsmeier et al. 2006) and Lepidoptera (Warren et al. 2021). Evidence for wild bees suggest that decline is a worldwide phenomenon (e.g. Zattara & Aizen 2021).

Pesticides have been repeatedly highlighted as a potential major driver of decline of wild insect pollinators (Vanbergen et al. 2013; Goulson et al. 2015; Potts et al. 2016; Dicks et al. 2021; Basu et al. 2024), with the decline of wild bee species closely correlating with pesticide use in the EU (Woodcock et al. 2016) and North America (Guzman et al. 2024). Recent field evidence underscores the sublethal impacts of pernicious pesticide use on wild bumble bees in USA (Richardson et al. 2024; Strang et al. 2024).

Current testing of pesticides on insect pollinators has focused on the managed honey bee (*Apis mellifera*), commercially available bumble bees (Europe: *Bombus terrestris*; USA: *Bombus impatiens*) and recently commercialised mason bees (Europe: *Osmia bicornis*; USA: *Osmia lignaria*) (Dirilgen et al. 2023). Wild bee species, particularly wild and solitary ground-nesting (fossorial) bee species, have more recently come into focus for ecotoxicological studies because of their importance for crop and wildflower pollination (Garibaldi et al. 2013) and the recent demonstration of significant negative pesticide impacts on their populations (Willis Chan & Raine 2021; Rondeau 2024; Rondeau & Raine 2024) and the potential for exposure of solitary fossorial bees to pesticides in agricultural landscapes (Willis Chan & Rondeau 2024).

The EU possesses three major wild insect pollinator taxa: bees (Hymenoptera), butterflies/moths (Lepidoptera) and syrphid flies (Diptera), which across the EU (and all other European countries) comprise over ~2,000 wild bee species, ~1,000 syrphid fly species and ~8,000 butterfly/moth species. It is likely that populations of all three are at risk from pesticides and therefore that all three taxa warrant risk assessment.

4.1 WildPosh contribution to ecotoxicological testing of wild pollinators

Indeed, there is growing awareness of the considerable variation across (and even within) insect species in their sensitivity to pesticides (Nagloo et al. 2024). This awareness has prompted repeated calls for ecotoxicological testing of a far wider range of wild bee species (Dietzsch & Jütte 2021; Topping et al. 2021; EFSA 2023; Jütte et al. 2023; Basu et al. 2024) so as to provide a more rigorous assessment of the risks posed by pesticides to wild insect pollinator populations.

An important step in ecotoxicological research and risk assessment is evaluation of an insect's sensitivity to a pesticide, for which protocols for non-model organisms are lacking (Jütte et al. 2023; Basu et al. 2024). WildPosh aims to fill this knowledge gap in the risks posed by pesticides for wild insect pollinators. Deliverable 2.4, reported herein, now

provides the essential protocols to allow a far wider range of wild insect pollinators (wild bee species, wild Lepidoptera species and wild hover fly species) to be employed in ecotoxicological testing.

5 Development of protocols

Based on recent literature arising from the PoshBee project that has made a concerted effort to develop protocols to assess the pesticide sensitivity of several wild bee (summarised in Barraud et al. 2022; Dewaele et al. 2024) and other recent literature e.g. for testing mason bee (*Osmia*) larvae to pesticides (Eeraerts et al. 2020), the WP2 Partners generated draft protocols for testing:

(i) wild bee species
(ii) wild Lepidoptera species
(iii) wild syrphid fly species

In the first 12 months of WildPosh, WP2 Partners have met once in person (GA/Kick-Off meeting on 25-26 Jan 2024), once at the on-line GA (3 July) and eight times on-line for dedicated discussion of the protocols (15 Jan, 22 Feb, 8 March, 13 March, 28 March, 11 April, 25 June, 11 Dec) as well as twice in joint meetings with the EU PollinERA project (16 Feb, 12 Sept). This intense schedule of meeting has allowed the WP2 Partners to develop workable protocols with amenable wild insect pollinators, informed by trial-and-error feedback from concurrent laboratory experiments at the respective Partner institutes.

All protocols form **Appendix 1**. Below we provide generic details to the protocols.

5.1 Choice of model species

The current list of model species has been expanded to 10 wild bee species, including 4 fossorial species, 2 mason bee species and 4 bumble bee species, including wild *B. terrestris*. A current (as of 31.12.2024) list of species is provided in M3 (delivery date 31.12.2024) and as **Appendix 2** to this report. In comparison to species listed in the original WildPosh proposal, it has replaced species that were difficult to maintain in the laboratory or that were not readily available from the field.

5.2 Comparison to the model species: *Bombus terrestris*

All protocols are initially foreseen to provide a relative measure of sensitivity to a pesticide by analysis of the response of a species in comparison to our reference model: the commercially available bumble bee *B. terrestris*. The dose used for a particular target species is the weight-adjusted LD50 of *B. terrestris* for the same life-stage (larval/pupal/adult) and mode exposure (contact/oral) for the three chosen pesticides: acetamiprid (insecticide), cypermethrin (insecticide) and tebuconazole (fungicide).

5.3 Chronic exposure

Sublethal effects of chronic exposure have repeatedly been highlighted as a lacuna in current ecotoxicological testing, which the WildPosh protocols aim to address. To do so, the experimental protocols allow extension to quantify chronic (long-term survival) and sublethal impacts (larvae: emergence success, fluctuating asymmetry (FA) of wing veins; adults: behavioural disruption) over a 10-day period of exposure. We are developing an ethogram, which is already incorporated into the protocols, to allow objective assessment of behavioural changes induced by pesticide exposure of adult target species. Protocols will be updated in 2025 to improve our rational, science-based protocols.

5.4 Life-stages

The protocols provide separate methods for assessing contact and oral pesticide exposure of both juvenile (larvae/pupae) and adult (imago female, imago male) stages of wild bees, Lepidoptera and syrphid flies. These are important because juvenile stages may prove more sensitive to pesticide exposure than adults and because juvenile stages may be more exposed to pesticides e.g. through contamination of soil for fossorial bees (Willis Chan & Raine 2021; Rondeau 2024; Rondeau & Raine 2024). Generating protocols for syrphid flies is highly innovative and has proved challenging. Adult testing has taken predominance and therefore a larval testing protocol has not yet been tested. We therefore do not provide a draft topical larval protocol for hover flies.

5.5 LD50 and data analysis

Protocols are easy to adapt to obtain LD50 estimates by using a range of pesticide concentrations (rather than the weight-adjusted LD50 dose of commercial *B. terrestris*). We note that many wild insect species may be difficult, or it may be ethically unjustified, to collect in large enough numbers to permit formal assessment of the LD50 of a species.

Protocols foresee the collection of a range of data related to mortality and sublethal effects (morphological and behavioural). Datasheets (**Appendix 3**) have been generated to simply data collection and R scripts (**Appendix 4**) have been developed to facilitate initial data processing (quality control).

5.6 Outlook

WP2 foresees continue testing of WildPosh protocols and wild insect pollinator species as well as generation of data on species sensitivity to pesticides during 2025 (2nd year of WildPosh). The protocols as provided represent current best practice and knowledge. With experience and data gained in 2025, protocols will be accordingly refined and updated.

Through the development and use of WildPosh protocols, we anticipate that the toxicological and ecological impacts of pesticides will be better understood and risk

assessments for relevant highly exposed species will be strengthened. Toward this end, in 2027 WildPosh Task 2.4 in cooperation with the PollinERA project aim to synthesise knowledge and experience across protocols so as to extend formal regulatory testing using wild EU/European insect pollinators. This will facilitate better assessment of pesticide effects (before approval) and improved protection from pesticide effects of Europe's wild insect pollinators.

6 Acknowledgements

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7 References

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8 Annex

Appendix 1. Protocols for testing the sensitivity of wild insect species to pesticides.

Appendix 2. Lists of wild pollinator species for ecotoxicological testing.

Appendix 3A. Datasheets for recording data generated by the protocol for acute oral exposure.

Appendix 3B. Datasheets for recording data generated by the protocol for acute topical exposure.

Appendix 4. R-scripts for initial processing of data generated by the protocols.

WildPosh D2.4: Appendix 1

Protocols for testing the sensitivity of wild insect species to pesticides.

BEES

Protocol for assessing pesticide acute oral toxicity to bee adults in controlled conditions

1. CONTEXT

1.1. Aim of the experiment

This experiment aims to orally and acutely expose wild adult bee females to two insecticides (Acetamiprid, Cypermethrin) and a fungicide (Tebuconazole), separately and in combination, to measure the acute toxicity of these products. This protocol is based on OECD guideline 247, Dewaele *& al*. (2024), Hellström *& al*. (2023) and Azpiazu *& al*. (2023).

It can be adapted for males.

1.2. General overview

Adult female bees (Table 1) are exposed to aqueous sucrose solution containing the test item. The test lasts at least 96 hours. Mortality is recorded daily at 4h, 24h, 48h, 72h and 96h and is compared with control values (Figures 1 and 2).

Table 1. Bee species to be tested within WildPosh (see D2.4, **Appendix 2** and Table 2 below).

Species	Laboratory	Females	Males	Period
Bombus terrestris	Mons	Jean Habay (breeder)	Microcolonies	May - June 2024
Bombus hypnorum	Mons	Jean Habay (breeder)	Microcolonies	June - August 2024
Bombus pascuorum	Mons	Jean Habay (breeder)	Microcolonies	June - August 2024
Bombus lapidarius	Mons	Jean Habay (breeder)	Microcolonies	June - August 2024

Andrena vaga	Mons	Field	Field	April - May 2025
Osmia bicornis	Freiburg	Cocoons from nesting aids	Cocoons from nesting aids	2024 (adult males and females; April-June)
Osmia brevicornis	Freiburg	Cocoons from nesting aids	Cocoons from nesting aids	2024 (adult females; April-June)
Anthophora plumipes	Halle	Cocoons from nest boxes	Cocoons from nest boxes	April-June 2025
Colletes hederae	Mons/Halle	Field	Field	August - October 2024/2025
Lasioglossum malachurum	Halle	Field	Field	2025

Fig. 1. Timeline oral acute exposure on solitary bee species females

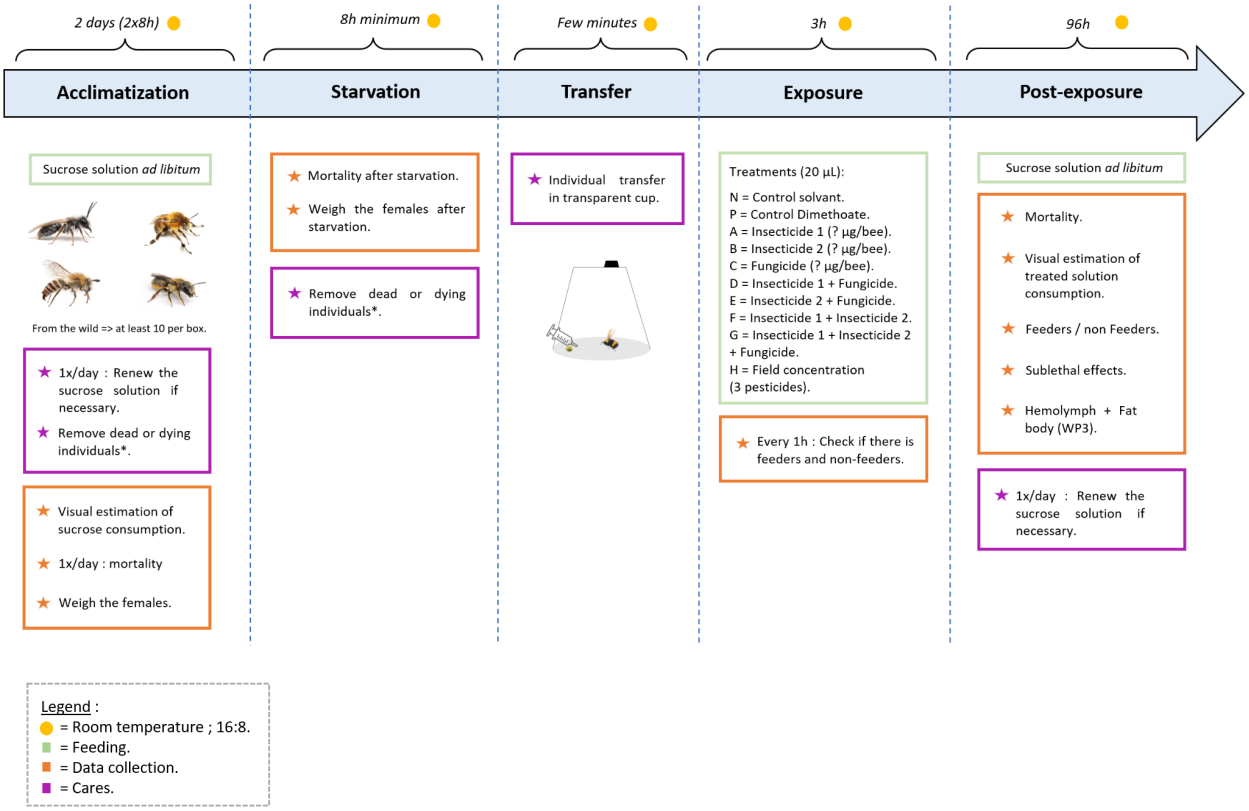
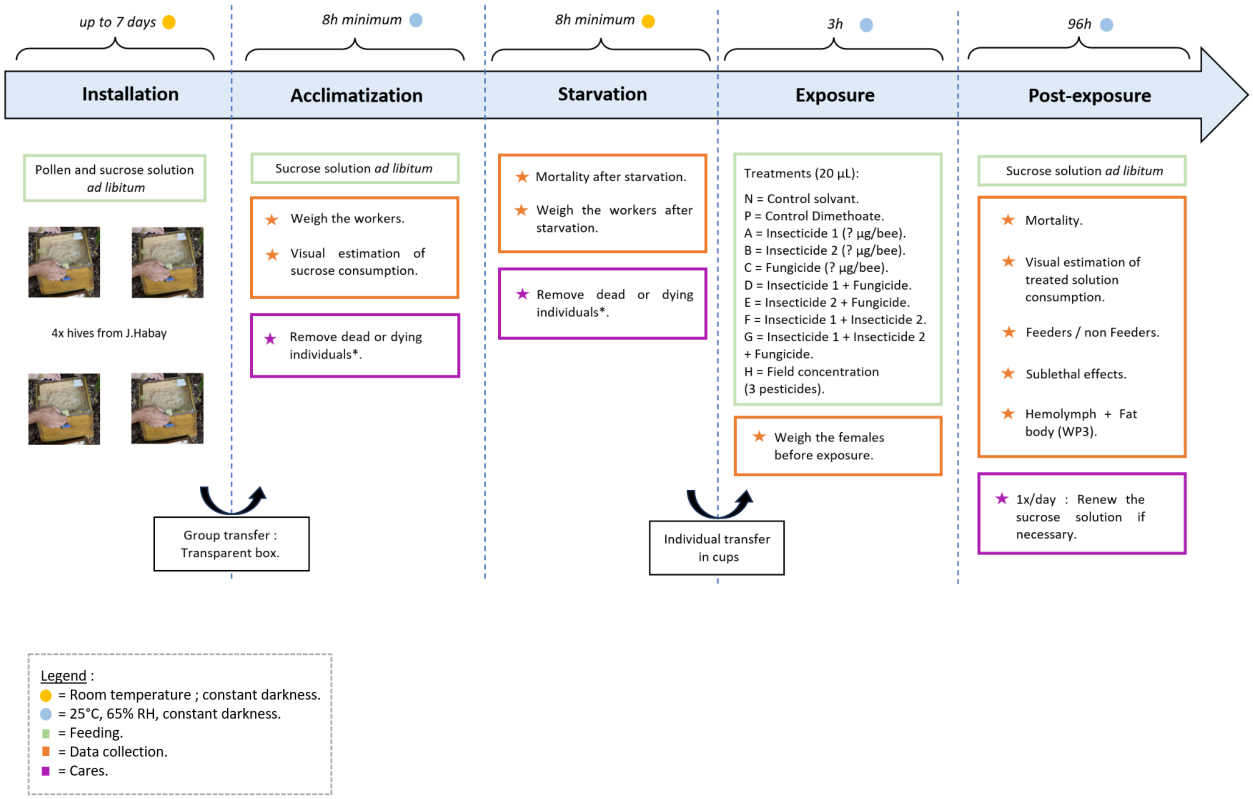


Fig. 2. Timeline oral acute exposure on social bee species workers



*Bees are unable to walk and show only very slight movements of their legs and antennae, reacting only weakly to stimulation, such as light or breath.

2. METHODS

2.1. Material preparation

Material	x
Adult from test species (Field or colonies), minimum 30 individuals per treatment	
Tweezers for handling test species	
Precision balance	
30 transparent boxes (for solitary bees) https://www.ikea.com/de/de/p/samla-box-transparent-70102972/	
400 transparent standard cups LATERN 50 Pièces Gobelets en Plastique PET Transparents Réutilisables, 700ml Très Résistants Verre à Pinte, Gobelets Boissons Froides pour Café Glacé, Dessert, Granité, Slurpee, BBQ : Amazon.fr: Cuisine et Maison	
Petal (feeding cue : adapted to the species) + double sided tape	
400 plastic transparent vials	
400 syringes (2mL)	
400 Eppendorfs (0,2 mL)	
Paper towel	
Sucrose solution: 1. Mineral water (infant water) 2. Sucrose	
Active Substances (provided by Umons if necessary) N-(6-Chloro-3-pyridylmethyl)-N-cyano-N-methylacetamidine PESTANAL , analytical standard 160430-64-8 (sigmaaldrich.com) N-(6-Chloro-3-pyridylmethyl)-N-cyano-N-methylacetamidine PESTANAL , analytical standard 160430-64-8 (sigmaaldrich.com) Tebuconazole PESTANAL , analytical standard 107534-96-3 (sigmaaldrich.com)	
Solvent (acetone)	
Dimethoate	

2.2. Acclimatization time

1. Collect solitary bee species from the field (in a pesticide-free natural environment, at the peak of emergence) or collect/order cocoons of solitary bees and incubate them (for *Osmia brevicornis* and *Osmia bicornis*). For social bees, obtain colonies from the breeder.
2. Prepare a sucrose solution according to the species needs (see table 2). Renew the solution two to three times a week if needed. Store the solution in the refrigerator.
3. Maintain individuals under laboratory conditions as described in the table 2 below during the full time of this step (Figure 3).
4. Place a 2mL syringe without a tip containing 2mL of sucrose solution inside the box.
5. Place the petal with tape at the end of the syringe (use a petal cue adapted to the species, important: note down flowering plant species used per bee species).
6. During acclimatization, check for the presence of dead or moribund individuals as well as the syrup consumption.
7. Stop acclimatization after 8h for the bumblebees, and 2 days (2x8h) for the solitary bees.

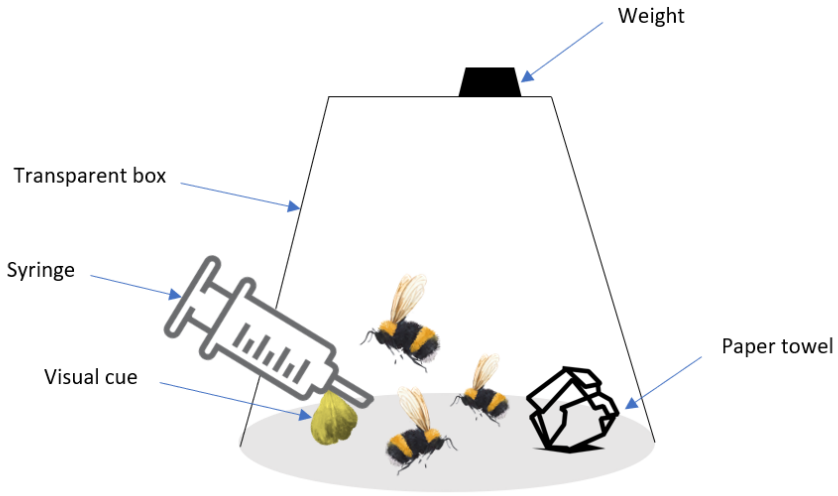
DATA to collect during acclimatization time:

1. Evaluate the average feeding behavior: measure (quantification from the syringe after 8h) the sucrose solution after 8h consumption (1x for bumblebees, 2x solitary bees). This data will be used for WP4 traits.
2. Record the number of individuals for each group, associated to their consumption.
3. Mortality of individuals (% of individuals dying during this time).

Table 2. Methods for maintaining species under controlled conditions :

Species	Storage	Conditions	Food
Bombus terrestris	Colonies	• 25°C • 65% RH • Constant dark	• Sucrose solution *ad libitum* (50%) • Pollen candy
Bombus hypnorum	Colonies	• 25°C • 65% RH • Constant dark	• Sucrose solution *ad libitum* (50%) • Pollen candy
Bombus pascuorum	Colonies	• 25°C • 65% RH • Constant dark	• Sucrose solution *ad libitum* (50%) • Pollen candy
Bombus lapidarius	Colonies	• 25°C • 65% RH • Constant dark	• Sucrose solution *ad libitum* (50%) • Pollen candy
Andrena vaga	Transparent box	• Room temperature • 16:8 (natural light)	• Sucrose solution *ad libitum* (50%) • Petal (feeding cue) : ?
Osmia bicornis	Transparent box	• Room temperature • 16:8 (natural light)	• Sucrose solution *ad libitum* (33%) • Petal (feeding cue): *Isatis tinctoria* (until end of Mai), then *Sinapis arvensis*
Osmia brevicornis	Transparent box	• Room temperature • 16:8 (natural light)	• Sucrose solution *ad libitum* (33%) • Petal (feeding cue): *Sinapis arvensis*
Anthophora plumipes	Transparent box	• Room temperature • 16:8 (natural light)	• Sucrose solution *ad libitum* (50%) • Petal (feeding cue) : ?
Colletes hederae	Transparent box	• Room temperature • 16:8 (natural light)	• Sucrose solution *ad libitum* (50%) • Petal (feeding cue) : ?
Lasioglossum malachurum	Transparent box	• Room temperature • 16:8 (natural light)	• Sucrose solution *ad libitum* (50%) • Petal (feeding cue) : ?

Fig. 3. Set up of a group box



2.3. Before exposure: preparation of solutions

Treatments (in order of priority):

- 1 treatment P: positive control dimethoate.
- 1 treatment N: negative control solvent (acetone).
- 1 treatment A: Acetamiprid.
- 1 treatment B: Cypermethrin.
- 1 treatment C: Tebuconazole.
- 1 treatment D: Acetamiprid + Cypermethrin.
- 1 treatment E: Acetamiprid + Tebuconazole.
- 1 treatment F: Cypermethrin + Tebuconazole.
- 1 treatment G: Tebuconazole - Acetamiprid - Cypermethrin.
- 1 treatment H: field concentration: combination of the three active ingredients (extreme values found on both apple and OSR fields).

Prepare stock solutions (**use acetone as a solvent for all the pesticides)** :

1. Weigh the active ingredient (AI) in a cup (be careful to use a glass cup rather than a plastic one, as acetamiprid crystals are difficult to recover). Use a high-accuracy scale to make sure that the very little amount of AI is as accurate as possible.
2. Place a funnel in a volumetric flask and rinse the contents of the cup with acetone to the mark.

3. Shake the solution by turning the volumetric flask upside down.
4. Pour the solution into a bottle and add the required volume of syrup.
5. Rinse the volumetric flask again with the final solution.
6. Mix the stock solution for at least 1 hour using a magnetic stirrer.

=> Prepare solution for each pesticide according to LD50 of bumblebees. Information on bumblebees LD50 provided by Manon:

Pesticides	Oral			Contact		
	LC50 (mg/L)	LD50 (µg a.i/bee)	LD50 (µg a.i/bee per g of bodyweight)	LC50 (mg/L)	LD50 (µg a.i/bee)	LD50 (µg a.i/bee per g of bodyweight)
Acetamiprid	1365,29	54,67	235,522	50 000	100	408
Cypermethrin	154,63	6,19	25,85	480,35	0,96	4,72
Tebuconazole	2500	100	408	50 000	100	408

=> Field concentration: No adjustment based on the weight. Summary information from Poshbee database and values proposed by Tomasz.

For acute toxicity testing - I recommend the 90th percentile,
for chronic toxicity - the median.

POSHBEE data					
Concentrations of pesticides (µg/kg) in:					
OSMIA pollen stores					
	Mean	Median	**P90**	Max	Percentage of samples with residues:
Acetamiprid	23.2	8.1	59.4	143	24%
Cypermethrin				1.4	2% (1 finding)
Tebuconazole	26.3	18.5	51.6	82.2	24%
BOMBUS pollen stores					
Acetamiprid	127	18.4	476	1090	27%
Cypermethrin	7.7	5.0	15.0	18.7	5%
Tebuconazole	31.6	14.4	88.8	189	41%
APIS beebread					
Acetamiprid	32.3	13.4	85.3	238	27%
Cypermethrin	6.1	3.2	12.0	14.2	2%
Tebuconazole	54.7	19.1	106	616	48%
TOTAL					
Acetamiprid	63.9	14.1	126	1090	26%
Cypermethrin	6.3	3.4	15.6	18.7	3%
Tebuconazole	41.9	18.4	88.5	616	39%

=> Prepare stock for each species according to the global average weight of the females / workers (fresh weight during acclimatization).

1. Calculate the mean body mass of the test species based on the weighing you performed.
2. Adjust the *B. terrestris* LD50 by multiplying it with the ratio of the mean body mass of the test species and the *B.terrestris* body mass, i.e. LD50-*B. terrestris* * (mean body mass test species/ mean body mass *B. terrestris*). For bees, LD50 should be adjusted for body mass separately for males and females due to their marked sexual size dimorphism.
3. Determine the v/v ratio of stock solution to sugar solution as follows: Create 0.02 mL aliquots that are later to be used for exposure of single bees and two 2 mL aliquots per treatment to be sent to PIWET.

The solutions should be well labelled.

Codes can be entered for this purpose:

Species_C_v/v ratio_S%_T_Date

Species – Bombus terrestris (BT), etc.
C – Stock Solution Concentration [µg/ml]
v/v ratio – v/v ratio of stock solution to sugar solution
S% – Sugar Solution Concentration [%]
T – Treatment Identifier (one of P, N, A, B, C, D, E, F, G, H)
Date – Date of solution preparation
Example: BT_800_1:1_50%_A_20240328

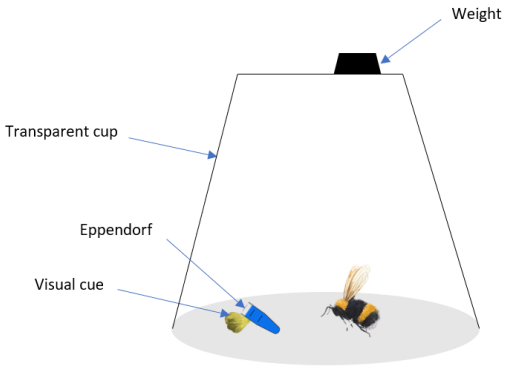
Store at least one aliquot of each of the feeding solutions at -20°C. Also keep the first and last steps of the dilution to validate the dosage.

2.4. Starvation, pesticide exposure and post-exposure

1. Keep all the time same conditions than acclimatization time (Table 2)
2. Starvation time (in group):
    - Social bees: From 2 to a maximum of 4 hours before exposure.
    - Solitary bees: Overnight at room temperature.

3. After starvation, put the females to sleep individually in transparent vials or nicot cages (in an ice tray with wadding) and measure the body mass of the individuals.
4. Then, place them under separate cups (Fig. 4).
5. Then assign them to various treatments in a semi-random manner, utilizing the designated R script to minimize body mass disparities between different treatments. Aim at obtaining a minimum of 30 alive and feeding individuals per treatment (therefore use at least 40 individuals per treatment or more, depending on the feeding rate from previous experiments). Since it may be difficult to manage this number of samples across all treatments at once it may be useful to run the experiment in batches i.e. first test a subset of replicates and then another subset of replicates. To avoid biases of testing period/hatching, it is better to test all treatments simultaneously but in batches of replicates than testing some treatments earlier than others. Afterwards, expose the individuals to treatments for 3h through a microtube (see Figure 4):
    - Social bees: 40 µL of treated solution.
    - Solitary bees: 20 µL of treated solution.
6. During exposure, check every 1h if bees are feeding (feeders / non-feeders). After three hours exclude non-feeders from the experiment. A non-feeder is a specimen that consumes < 80 % of the mean consumption of the respective treatment group within the maximum exposure (feeding) period.
7. After exposure, leave the females in their individual cages.
8. Observe individuals for 4h, 24h, 48h, 72h and 96 h.

Fig. 4. Set up of the exposure cages (cups)



DATA to collect during starvation, exposure time and post-exposure time

1. Individual body mass after starvation time
2. Consumption of treated syrup during exposure (visual evaluation if 50% is consumed) -> classification feeders and non-feeders.
3. Mortality checks at: 4h; 24h; 48h; 72h and 96h.
4. Sublethal effects (qualitative measurements): tremor; paralysis; reduced coordination. Sublethal effects should be recorded with the terminology in the ethogram below (sent by Simone, please keep it confidential). Behavior not included in the ethogram should be carefully described and/or recorded on video for posterior discussion.

Behavior	**Description**	**Video (link)**
Still	The bee stands on its legs, without exhibiting any active behavior (i.e., walking, flying, or feeding).	To be provided/species
Self-grooming	The bee rubs its legs on its body, typically against the head, the abdomen, and/or other legs. When touching the head, the bee typically rubs the mouthparts, the eyes, or its surroundings, and/or the antennae. When touching the abdomen, the bee typically rubs its ventral sides.	To be provided/species

Feeding	The bee approaches the feeder, unfolds its proboscis, and inserts it in the syringe to imbibe the sucrose solution.	To be provided/species
Self-amputation	The bee removes at least one of its own legs.	To be provided/species
Walking	The bee walks actively, exhibiting coordinated movements. No falling behavior is observed.	To be provided/species
Flying	The bee performs coordinated flight using its wings, with all legs off the ground. The flight might consist of horizontally/vertically moving from one point to another or being steady.	To be provided/species
Falling	The bee attempts to walk on the vertical or top panel of the cage but loses its legs' adhesion to the surface and falls.	To be provided/species
Hyperactive	The bee manifests signs of excitation through chaotic and rapid expression of multiple behaviors, including walking, self-grooming, flight attempts, and/or falling. Overall, the bee manifests poor coordination.	To be provided/species
Weak	The bee may be actively moving, still, or laying on one side. The bee movements, occurring spontaneously or after stimulation, are characterized by poor coordination and slowness.	To be provided/species
Thanatosis	The bee lays on the ground, typically on its back, with no active movement of its body. The bee exhibits regular ventilatory cycles and may move the antennae. Upon stimulation, the bee reacts and then moves actively exhibiting coordinated movements.	To be provided/species

Moribund	The bee lays on the ground with no active movement of its body, typically laying on one side or on its back. The bee exhibits regular ventilatory cycles and may move the antennae. Upon stimulation, the bee does not actively react.	To be provided/species
Dead	The bee is immobile and does not react upon stimulation. The abdomen and antennae do not exhibit spontaneous movements.	To be provided/species

The ethogram above will be adjusted for each taxonomic group and potential for each species used.

2.5. Treatment of specimens after experiment

Preparation of labels for samples. To be specified.

For WP3, collect haemolymph and fat bodies for proteome studies and for transcriptome studies, preferably from the same specimens, using specimens living at 48 hrs post treatment. Details are to be provided in protocols from WP3.
For the proteomics analysis, collect n=15 haemolymph samples per treatment and take from the same bees, the corresponding fat bodies (start with sampling haemolymph prior to the fat bodies). For transcriptomics, collect a minimum of n = 8 fat bodies (bees will be killed by snap freezing in liquid nitrogen and dissected in RNAlater to isolate the fat bodies. Those ones will be stored in RNAlater and immediately frozen in -80°C. Bear in mind to not use the same individuals for the haemolymph (proteomics), and fat body samples (transcriptomics) as a stressful haemolymph sampling will change the transcriptome. Haemolymph and fat bodies will be taken from the same individual.
The most sensitive species will initially be studied.

In case of *Bombus terrestris* at each stage of mortality check, collect dead individuals separately to Eppendorf tubes marked with a specific code (Species_Mass_T_MC_XX), freeze and send to PIWet for toxicokinetic analysis.
Codes should be entered as **Species_Mass_T_MC_XX**
where:
Species – Bombus terrestris (BT)
Mass – mass of the individual insect before pesticides exposure (in g)
T – Treatment Identifier (P, N, A, B, C, D, E, F, G or H)
MC – Mortality Check (4h, 24h, 48h, 72h or 96h)
XX – Amount of treated solution (µL)
Individuals after haemolymph collection also send to PIWet including additional mark

on the label (H): Species_ Mass_T_MC_XX**_H**
Individuals after fat bodies collection will not be tested by PIWet.

2.6. Raw data for toxicology

1. ID_unique = Code composed of Stage, Species, Sex and ID separated by underscore (automatically done by the excel file)

2. Stage = Adult (A); Larva (L).

3. Species = Test species: *Bombus terrestris* (BT); *Bombus hypnorum* (BH); *Bombus lapidarius* (BL); *Bombus pascuorum* (BP); *Andrena vaga* (AV); *Colletes hederae* (CH); *Anthophora plumipes* (AP); *Osmia brevicornis* (OBR); *Osmia bicornis* (OBI); *Lasioglossum malachurum* (LM).

4. Sex = Female (F); Male (M).

5. ID = Number of individual unique within the same stage of the same species of the same sex but not across different species/stages/sexes: 1, 2, 3, 4

6. Origin = Origin of specimen. This could be either a site or the vender (in the case of Osmia)

7. Date_obtained = The date it was collected from the wild as adults or hatched from cocoons

8. Date_pre_assessment = The date the pre-assessment was done (i.e. determination of Body_mass, SC_pre_expo, Dead_pre_expo)

9. Age_pre_expo = Difference between Date_pre_assessment and Date_obtained in days

10. Body_mass = Body mass of the individual at the start of the experiment (in g)

11. Dead_pre_expo = Alive (0); Dead (1) in pre-assessment

12. Sublethal_effs_pre_expo = Sublethal effects before exposure

13. Excluded_manually = Excluded (1) or not excluded (0 or blank) based on decision by file manager rather than the R script on treatment allocation

14. Observer = Person taking the measurements

15. Data_enterer = Person entering data in this excel file

16. Comments = Comments, e.g. details on sublethal effects

Post-exposure data (at individual level):

1. Treatment = "P: positive control dimethoate. N: negative control solvent (acetone). A: Acetamiprid. B: Cypermethrin. C: Tebuconazole. D: Acetamiprid + Cypermethrin. E: Acetamiprid + Tebuconazole. F: Cypermethrin + Tebuconazole. G: Cypermethrin + Tebuconazole + Acetamiprid. H: field concentration (extreme value)."

2. Stage = Adult (A); Larva (L).

3. Species = Test species: *Bombus terrestris* (BT); *Bombus hypnorum* (BH); *Bombus lapidarius* (BL); *Bombus pascuorum* (BP); *Andrena vaga* (AV); *Colletes hederae* (CH); *Anthophora plumipes* (AP); *Osmia brevicornis* (OBR); *Osmia bicornis* (OBI); *Lasioglossum malachurum* (LM).

4. Sex = Female (F); Male (M).

5. ID = Number of individual unique within the same stage of the same species of the same sex but not across different species/stages/sexes: 1, 2, 3, 4

6. Date_exposure = Date of exposure

7. T_syrup_offered = Volume of treated syrup offered in ml

8. TSC = Treated syrup consumption as a proportion of the whole dose (visually estimated). Value between 0 and 1

9. Dead_after_4h = Alive (0); Dead (1) 4 hours after exposure

10. Sublethal_effs_4h = Sublethal effects 4 hours after exposure

11. Dead_after_24h = Alive (0); Dead (1) 24 hours after exposure

12. Sublethal_effs_24h = Sublethal effects 24 hours after exposure

13. Dead_after_48h = Alive (0); Dead (1) 48 hours after exposure

14. Sublethal_effs_48h = Sublethal effects 48 hours after exposure

15. Dead_after_96h = Alive (0); Dead (1) 96 hours after exposure

16. Sublethal_effs_96h = Sublethal effects 96 hours after exposure

17. SC_after_96h = Total syrup consumption after exposure

3. CHECKLIST

3.1. Solitary bee species

Schedule	***Protocol***	**×**
Day 1 (……)	**Preparation of sucrose solution** **Reception and storage of females** ● Place the individuals in a room at room temperature in boxes (10 females per box). ● Feed them with sucrose *ad libitum* (with petal cue).	
Day 2 (……) **9h**	**Daily checks :** ● Checking and removing dead or dying individuals (Mortality). ● Renew the sucrose solution if necessary (Visual estimation of sucrose consumption).	
Day 3 (……) **9h** **13h** **18h**	**Daily checks :** ● Checking and removing dead or dying individuals (Mortality). ● Renew the sucrose solution if necessary (Visual estimation of sucrose consumption). **Preparation of feeding solutions** ● Final treated or control solutions (dilutions). **Remove the sucrose syringes and starve the individuals overnight at room temperature.**	
Day 4 (……) **8h** **8h30**	**Checking and removing dead or dying individuals** **Exposure of individuals** ● Weigh the females individually. ● Place them in an individual cup. ● Place 20µL of treated solution in a lid (with petal cue).	

9h30 / 11h30 **11h30** **15h30**	**<u>Check every 1h if bees are feeding (feeders and non-feeders).</u>** **<u>Withdrawal of treatments</u>** ● Visual check of syrup consumption and feeders or non-feeders. ● Leave the females in the individual cage. **<u>Check</u>** ● Mortality. ● Sublethal effects.	
Day 4 (……) ***Day 5 (……)*** ***Day 6 (……)*** ***Day 7 (……)***	**<u>Check</u>** ● Mortality. ● Sublethal effects.	

3.2. Social bee species

Schedule	***Protocol***	**×**
Day 1 (……)	**<u>Preparation of sucrose solution</u>** **<u>Reception and storage of colonies</u>** ● Place the colonies in a room at 20°C and 65% RH. ● Feed them with sucrose *ad libitum* and pollen candy.	
Days 3, 5 (……) **9h**	**<u>Daily checks :</u>** ● Add a pollen candy. ● Renew the sucrose solution if necessary (Visual estimation of sucrose consumption).	
Day 7 (……) **9h**	**<u>Reception and storage of females</u>** ● Place the individuals in a room at 20°C and 65% RH (10 females per box).	

	● Feed them with sucrose *ad libitum*.	
13h	**<u>Preparation of feeding solutions</u>** ● Final treated or control solutions (dilutions).	
Day 8 (……)		
8h	**<u>Daily checks :</u>** ● Checking and removing dead or dying individuals (Mortality). ● Renew the sucrose solution if necessary (Visual estimation of sucrose consumption).	
8h30	**<u>Starve the females.</u>**	
11h30	**<u>Exposure of individuals</u>** ● Weigh the females individually. ● Place them in an individual cup. ● Place 40µL of treated solution in a lid (with petal cue).	
12h30/13h30	**<u>Check every 1h if bees are feeding (feeders and non-feeders).</u>**	
14h30	**<u>Withdrawal of treatments.</u>** ● Visual check of syrup consumption and feeders or non-feeders. ● Leave the females in the individual cage.	
18h30	**<u>Check</u>** ● Mortality. ● Sublethal effects.	
Day 9 (……) ***Day 10 (……)*** ***Day 11 (……)*** ***Day 12 (……)***	**<u>Check</u>** ● Mortality. ● Sublethal effects. **<u>Daily checks :</u>** ● Checking and removing dead or dying individuals (Mortality). ● Renew the sucrose solution if necessary (Visual estimation of sucrose consumption).	

Protocol for assessing pesticide acute topical toxicity in the lab

1. CONTEXT

1.1. Aim of the experiment

The aim of this experiment is to topically and acutely expose wild bee females to two insecticides (Acetamiprid, Cypermethrin) and a fungicide (Tebuconazole), separately and in combination, to measure the acute toxicity of these products. This protocol is based on OECD guideline 247, Dewaele & al. (2024), Hellström & al. (2023) and Azpiazu & al. (2023)

It can be adapted for males.

1.2. General overview

Adult female bees are exposed to a treated or control solution on their thorax and are fed with an untreated sucrose solution. The test lasts at least 96 hours. Mortality is recorded daily at 4h, 24h, 48h, 72h and 96h and is compared with control values (Figures 1 and 2).

Table 1. Selection of bee species:

Species	Laboratory	Females	Males	Period
Bombus terrestris	Mons	Jean Habay (breeder)	Microcolonies	May - June 2024
Bombus hypnorum	Mons	Jean Habay (breeder)	Microcolonies	June - August 2024
Bombus pascuorum	Mons	Jean Habay (breeder)	Microcolonies	June - August 2024
Bombus lapidarius	Mons	Jean Habay (breeder)	Microcolonies	June - August 2024
Andrena vaga	Mons	Field	Field	April - May 2025
Osmia bicornis	Freiburg	Cocoons from	Cocoons from	2024 (adult males

		nesting aids	nesting aids	and females; April-June)
Osmia brevicornis	Freiburg	Cocoons from nesting aids	Cocoons from nesting aids	2024 (adult females; April-June)
Anthophora plumipes	Halle	Cocoons from nest boxes	Cocoons from nest boxes	April-June 2025
Colletes hederae	Mons/Halle	Field	Field	August - October 2024/2025
Lasioglossum malachurum	Halle	Field	Field	2025

Fig. 1. Timeline topical acute exposure on solitary bee species

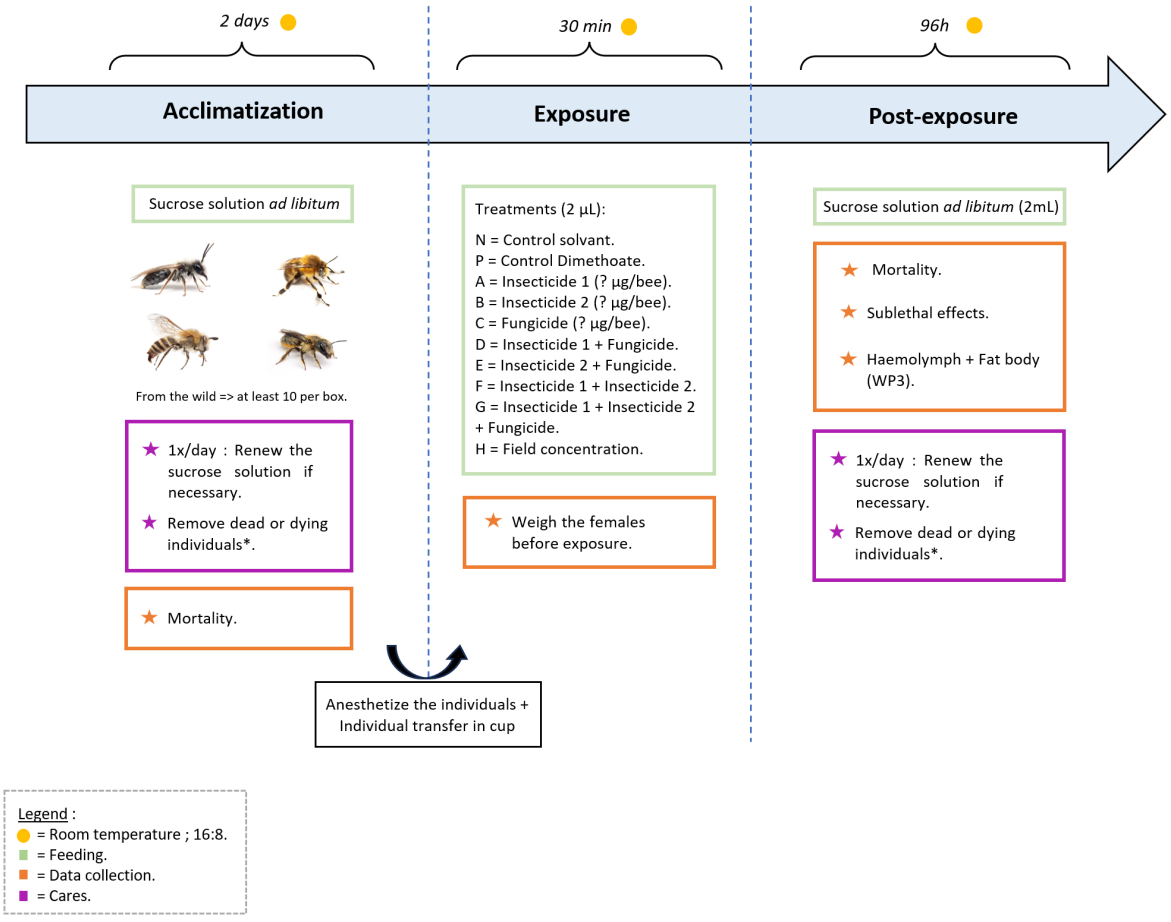
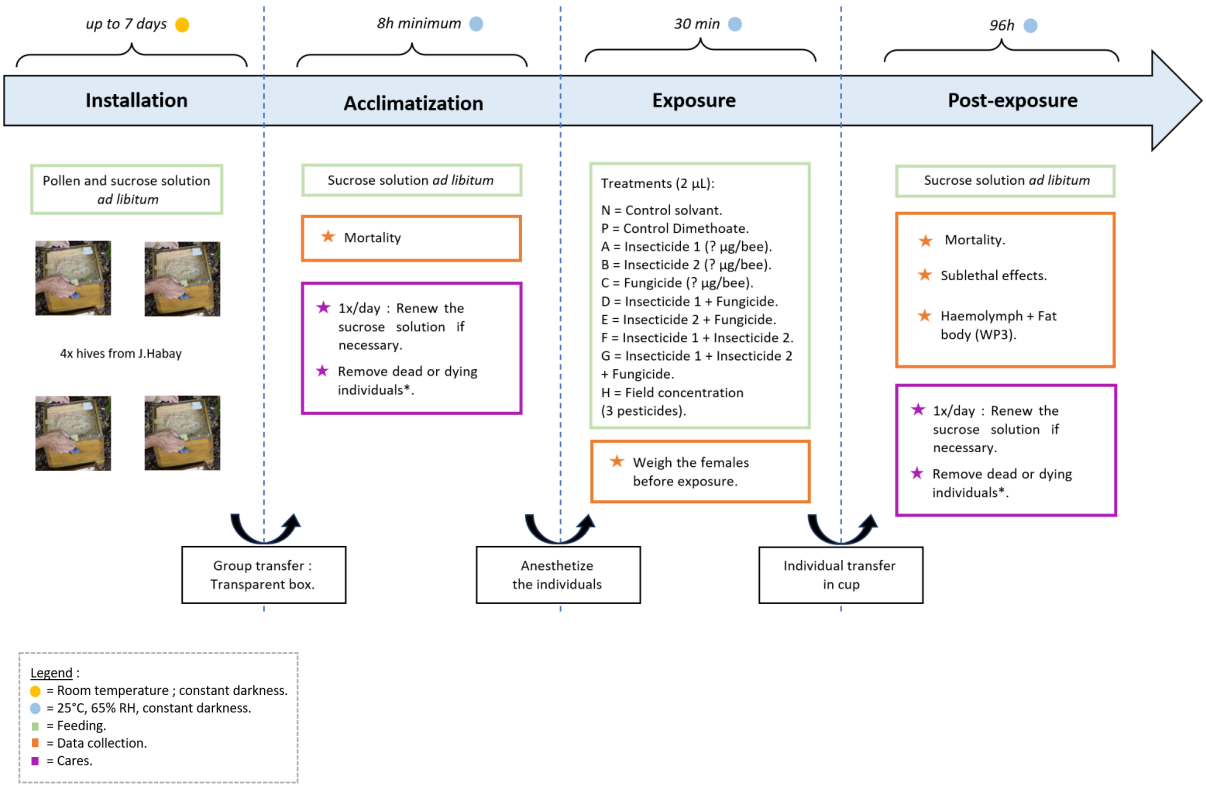


Fig. 2. Timeline topical acute exposure on social bee species workers



2. METHODS

2.1. Material preparation

Material	x
Test species (Field or colonies)	
Tweezers for handling test species	
Precision balance	
400 transparent standard cups LATERN 50 Pièces Gobelets en Plastique PET Transparents Réutilisables, 700ml Très Résistants Verre à Pinte, Gobelets Boissons Froides pour Café Glacé, Dessert, Granité, Slurpee, BBQ : Amazon.fr: Cuisine et Maison	
30 transparent boxes https://www.ikea.com/de/de/p/samla-box-transparent-70102972/	
Petal (feeding cue, adapted to the species) + double-sided tape	
400 plastic transparent vials	
400 syringes (2mL)	
400 standard cups	
10 micro-applicators	
Paper towel	
Sucrose solution: 1. Mineral water (infant water) 2. Sucrose	
Active Substances (provided by Umons if necessary) N-(6-Chloro-3-pyridylmethyl)-N-cyano-N-methylacetamidine PESTANAL , analytical standard 160430-64-8 (sigmaaldrich.com) N-(6-Chloro-3-pyridylmethyl)-N-cyano-N-methylacetamidine PESTANAL , analytical standard 160430-64-8 (sigmaaldrich.com) Tebuconazole PESTANAL , analytical standard 107534-96-3 (sigmaaldrich.com)	
Solvent (acetone)	
Triton X	
Dimethoate	

2.2. Reception of species and acclimatization

1. Collect solitary bee species from the field (in a pesticide-free natural environment, at the peak of emergence) or collect/order cocoons of solitary bees and incubate them (for *Osmia brevicornis* and *Osmia bicornis*). For social bees, obtain colonies from the breeder concerned.
2. Prepare a sucrose solution according to the species' needs (renew it two to three times a week if needed). Store the solution in the refrigerator.
3. Maintain individuals under laboratory conditions as described in the table below.
4. Place a 2mL syringe without tip containing 2mL of sucrose solution on the side of the box (beforehand, make a hole on the side of the box to insert the syringe).
5. Place the petal with tape at the end of the syringe (use a petal cue according to the bee species) use the same cue for all species).
6. Maintain the individuals in the conditions defined in table 2.
7. During acclimatization, check for the presence of dead or moribund individuals as well as the syrup consumption.
8. Stop acclimatization after 8h for the bumblebees, and 2 days for the solitary bees.

DATA to collect during acclimatization time:

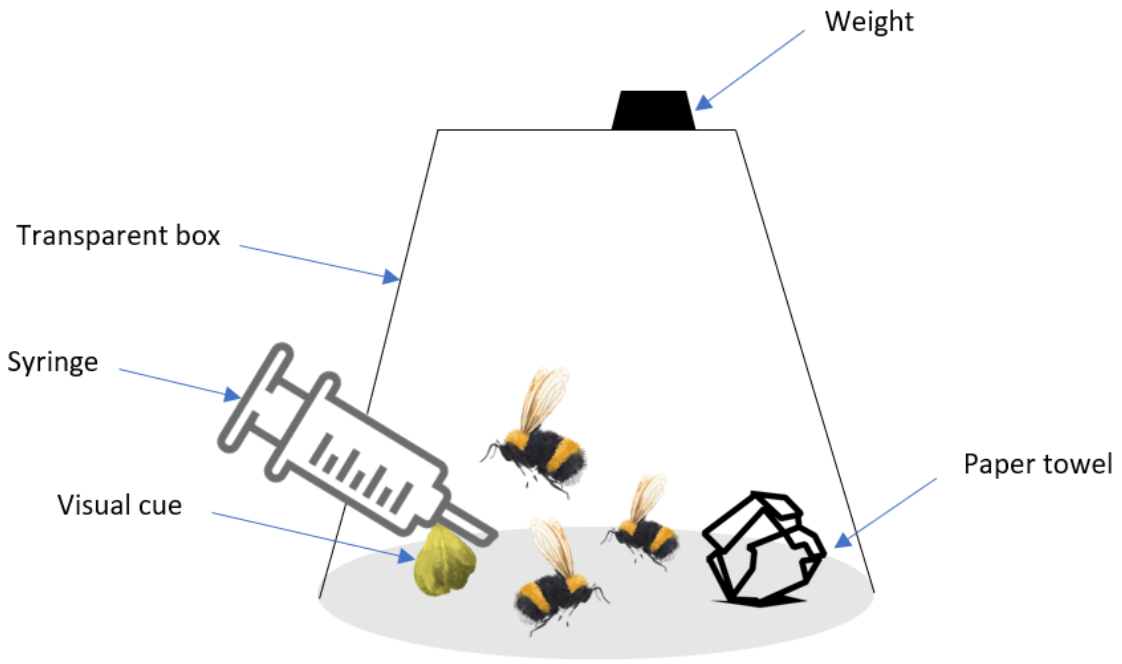
1. Evaluate the feeding behavior: measure visually the sucrose solution after consumption to see if females feed.
2. Mortality of specimens (% of individuals dying during this time).

Table 2. Methods for maintaining species under controlled conditions :

Species	Storage	Conditions	Food
Bombus terrestris	Colonies	• 25°C • 65% RH • Constant dark	• Sucrose solution *ad libitum* (50%) • Pollen candy
Bombus hypnorum	Colonies	• 25°C • 65% RH • Constant dark	• Sucrose solution *ad libitum* (50%) • Pollen candy
Bombus pascuorum	Colonies	• 25°C • 65% RH • Constant dark	• Sucrose solution *ad libitum* (50%) • Pollen candy
Bombus lapidarius	Colonies	• 25°C • 65% RH • Constant dark	• Sucrose solution *ad libitum* (50%) • Pollen candy

Andrena vaga	Transparent box	• Room temperature • 16:8 (natural light)	• Sucrose solution *ad libitum* (50%) • Petal (feeding cue)
Osmia bicornis	Transparent box	• Room temperature • 16:8 (natural light)	• Sucrose solution *ad libitum* (33%) • Petal (feeding cue)
Osmia brevicornis	Transparent box	• Room temperature • 16:8 (natural light)	• Sucrose solution *ad libitum* (33%) • Petal (feeding cue)
Anthophora plumipes	Transparent box	• Room temperature • 16:8 (natural light)	• Sucrose solution *ad libitum* (50%) • Petal (feeding cue)
Colletes hederae	Transparent box	• Room temperature • 16:8 (natural light)	• Sucrose solution *ad libitum* (50%) • Petal (feeding cue)
Lasioglossum malachurum	Transparent box	• Room temperature • 16:8 (natural light)	• Sucrose solution *ad libitum* (50%) • Petal (feeding cue)

Fig. 3. Set up of a group box



2.3. Preparation of solutions before exposure

Treatments (in order of priority):

- 1 treatment P: positive control dimethoate.
- 1 treatment N: negative control solvent (acetone).
- 1 treatment A: Acetamiprid.
- 1 treatment B: Cypermethrin.
- 1 treatment C: Tebuconazole.
- 1 treatment D: Acetamiprid + Cypermethrin.
- 1 treatment E: Acetamiprid + Tebuconazole.
- 1 treatment F: Cypermethrin + Tebuconazole.
- 1 treatment G: Cypermethrin + Tebuconazole + Acetamiprid .
- 1 treatment H field concentration (extreme value).

Prepare stock treatment solutions (**use the acetone as solvent for all the pesticides**):

1. Weigh the active ingredient (AI) in a cup (be careful to use a glass cup rather than a plastic one, as acetamiprid crystals are difficult to recover). Use a high-accuracy scale to make sure that the very little amount of AI is as accurate as possible.
2. Place a funnel in a volumetric flask and rinse the contents of the cup with acetone to the mark.
3. Shake the solution by turning the volumetric flask upside down.
4. Pour the solution into a bottle and add the required volume of syrup.
5. Rinse the volumetric flask again with the final solution.
6. Mix the stock solution for at least 1 hour using a magnetic stirrer.

Prepare treatment solutions:

- Dilute a volume of stock solution in acetone.
- Add 0.1% Triton X-100 to the solution serving as surfactant. Water control must contain the same amount of surfactant (Triton X-100) as the test item treatments.

=> Prepare solution for each pesticide according to LD50 of bumblebees. Bumblebees LD50 values provided by Manon.

Pesticides	Oral			Contact		
	LC50 (mg/L)	LD50 (µg a.i/bee)	LD50 (µg a.i/bee per g of bodyweight)	LC50 (mg/L)	LD50 (µg a.i/bee)	LD50 (µg a.i/bee per g of bodyweight)
Acetamiprid	1365,29	54,67	235,522	50 000	100	408
Cypermethrin	154,63	6,19	25,85	480,35	0,96	4,72
Tebuconazole	2500	100	408	50 000	100	408

=> Field concentration: No adjustment based on the weight. Summary information from Poshbee database and values proposed by Tomasz.

For acute toxicity testing - I recommend the 90th percentile,
for chronic toxicity - the median.

POSHBEE data					
Concentrations of pesticides (µg/kg) in:					
OSMIA pollen stores					
	Mean	Median	**P90**	Max	Percentage of samples with residues:
Acetamiprid	23.2	8.1	59.4	143	24%
Cypermethrin				1.4	2% (1 finding)
Tebuconazole	26.3	18.5	51.6	82.2	24%
BOMBUS pollen stores					
Acetamiprid	127	18.4	476	1090	27%
Cypermethrin	7.7	5.0	15.0	18.7	5%
Tebuconazole	31.6	14.4	88.8	189	41%
APIS beebread					
Acetamiprid	32.3	13.4	85.3	238	27%
Cypermethrin	6.1	3.2	12.0	14.2	2%
Tebuconazole	54.7	19.1	106	616	48%
TOTAL					
Acetamiprid	63.9	14.1	126	1090	26%
Cypermethrin	6.3	3.4	15.6	18.7	3%
Tebuconazole	41.9	18.4	88.5	616	39%

1. Calculate the mean body mass of the test species based on the weighing you performed.
2. Adjust the *B. terrestris* LD50 by multiplying it with the ratio of the mean body mass of the test species and the *B.terrestris* body mass, i.e. LD50-*B. terrestris* * (mean body mass test species/ mean body mass *B. terrestris*). For bees, LD50 should be adjusted for body mass separately for males and females due to their marked sexual size dimorphism.

3. Determine the v/v ratio of stock solution to sugar solution as follows: Create 0.2 mL aliquots that are later to be used for exposure of single bees and two 2 mL aliquots per treatment to be sent to PIWET.

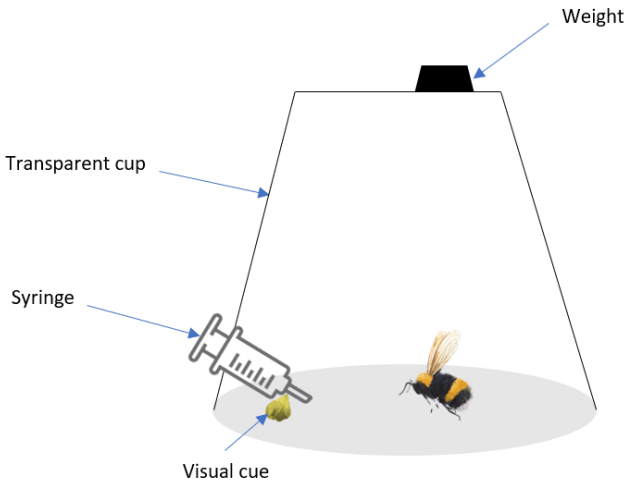
Store at least one aliquot of each of the feeding solutions at -20°C. Also keep the first and last steps of the dilution to validate the dosage.

2.4. Pesticide exposure

1. Calm the workers in transparent vials / nicot cage (in an ice tray with wadding). Be careful to not stress the specimen too long with the cold.
2. After measuring the body mass of the individuals, place them under separate cups (Fig. 4).
3. Then assign them to various treatments in a semi-random manner, utilizing the designated R script to minimize body mass disparities between different treatments. Aim at obtaining a minimum of 30 alive and feeding individuals per treatment (therefore use at least 40 individuals per treatment or more, depending on the feeding rate from previous experiments). Since it may be difficult to manage this number of samples across all treatments at once it may be useful to run the experiment in batches i.e. first test a subset of replicates and then another subset of replicates. To avoid biases of testing period/hatching, it is better to test all treatments simultaneously but in batches of replicates than testing some treatments earlier than others. Ensure each treatment group includes at least 40 individuals, with a goal of maintaining a minimum of 30 individuals per group, taking into account the feeding rate observed in prior experiments.
4. Expose the individuals to 2µL of the treatments on their thorax.
   => Use the adapted solution of 2µL drop according to the size of the species.

5. Wait for them to wake up in the vial and place them in an individual cage (Figure 4).
6. Observe isolated individuals for 96h under the cup.

Fig. 4. Set up of the post-exposure cages (cups)



DATA to collect during exposure time and post-exposure time

1. Body mass (after the chiling, before the exposure)
2. Mortality checks at: 4h; 24h; 48h; 72h and 96h.
3. Sublethal effects: unusual behavior; paralysis; reduced coordination. Sublethal effects should be recorded with the terminology in the ethogram below (sent by Simone, please keep it confidential). Behavior not included in the ethogram should be carefully described and/or recorded on video for posterior discussion.

Behaviour	**Description**	**Video (link)**
Still	The bee stands on its legs, without exhibiting any active behavior (i.e., walking, flying, or feeding).	To be provided/species
Self-grooming	The bee rubs its legs on its body, typically against the head, the abdomen, and/or other legs. When touching the head, the bee typically rubs the mouthparts, the eyes, or its surroundings, and/or the antennae. When touching the abdomen, the bee typically rubs its ventral sides.	To be provided/species

Feeding	The bee approaches the feeder, unfolds its proboscis, and inserts it in the syringe to imbibe the sucrose solution.	To be provided/species
Self-amputation	The bee removes at least one of its own legs.	To be provided/species
Walking	The bee walks actively, exhibiting coordinated movements. No falling behavior is observed.	To be provided/species
Flying	The bee performs coordinated flight using its wings, with all legs off the ground. The flight might consist of horizontally/vertically moving from one point to another or being steady.	To be provided/species
Falling	The bee attempts to walk on the vertical or top panel of the cage but loses its legs' adhesion to the surface and falls.	To be provided/species
Hyperactive	The bee manifests signs of excitation through chaotic and rapid expression of multiple behaviors, including walking, self-grooming, flight attempts, and/or falling. Overall, the bee manifests poor coordination.	To be provided/species
Weak	The bee may be actively moving, still, or laying on one side. The bee movements, occurring spontaneously or after stimulation, are characterized by poor coordination and slowness.	To be provided/species
Thanatosis	The bee lays on the ground, typically on its back, with no active movement of its body. The bee exhibits regular ventilatory cycles and may move the antennae. Upon stimulation, the bee reacts and then moves actively exhibiting coordinated movements.	To be provided/species

Moribund	The bee lays on the ground with no active movement of its body, typically laying on one side or on its back. The bee exhibits regular ventilatory cycles and may move the antennae. Upon stimulation, the bee does not actively react.	To be provided/species
Dead	The bee is immobile and does not react upon stimulation. The abdomen and antennae do not exhibit spontaneous movements.	To be provided/species

The ethogram above will be adjusted for each taxonomic group and potential for each species used.

For WP3, collect haemolymph and fat bodies for proteome studies and for transcriptome studies, preferably from the same specimens, using specimens living at 48 hrs post treatment. Details are to be provided in protocols from WP3. Collect a minimum of n=3 haemolymph samples per treatment and n = 8 fat body samples per treatment (do not use the same individuals for the haemolymph and fat body samples as a stressful haemolymph sampling will change the transcriptome).The most sensitive species will initially be studied.

2.5. Treatment of specimens after experiment

Preparation of labels for samples: To be specified.

For WP3, collect haemolymph and fat bodies for proteome studies and for transcriptome studies, preferably from the same specimens, using specimens living at 48 hrs post treatment. Details are to be provided in protocols from WP3. Collect a minimum of n=3 haemolymph samples per treatment and n = 8 fat body samples per treatment (do not use the same individuals for the haemolymph and fat body samples as a stressful haemolymph sampling will change the transcriptome). The most sensitive species will initially be studied.

For haemolymph collection, use the protocol described in the end of the following video: https://www.youtube.com/watch?v=bjzQVN7uGyg&t=40s&ab_channel=PoshBeeProject

The specimens are conserved with individual labels in an Eppendorf, in the freezer, for trait analyses (WP4, WP5). List of traits to be provided.

2.6. Raw data

Data :

1. ID = Identification.
    - Treatment = O; N; P; A; B; C; D; E; F; G.
    - Stage = Adult (A) ; Larva (L).
    - Sex = Female (F) ; Male (M).
    - Individual number = 01 ; 02 ; …
    - Collection site.
    - Species = Test species: *Bombus terrestris* (BT); *Bombus hypnorum* (BH); *Bombus lapidarius* (BL); *Bombus pascuorum* (BP); *Andrena vaga* (AV); *Colletes hederae* (CH); *Anthophora plumipes* (AP); *Osmia brevicornis* (OBR); *Osmia bicornis* (OBI); *Lasioglossum malachurum* (LM).
    - Team : Freiburg (F) ; Mons (M) ; Halle (H).
2. Day of collect (for wild species) / Hatching date (for Osmia).
3. Treatment = O; N; P; A ; B ; C ; D ; E ; F ; G.
4. Body mass = Body mass of the individual at the start of the experiment (in g).
5. SCbE = Syrup consumption before exposure.
6. Date of exposure.
7. Hour of death.
8. Mortality = Alive (0) ; Dead (1).
9. Sublethal effects = Yes (1) ; No (0).
10. Feeder = Feeder (0) ; Non-Feeder (1).
11. Description of sublethal effects.
12. Removed before treatment (if it is the case) = Yes (1) ; No (0).
13. Box allocation = Box number of the treatment.

3. CHECKLIST

3.1. Solitary bee species

Schedule	***Protocol***
Day 1 (……)	**Preparation of sucrose solution** **Reception and storage of females** • Place the individuals in a room at room temperature in boxes (10 females per box). • Feed them with sucrose *ad libitum* (with petal cue).
Day 2 (……) **9h**	**Daily checks :** • Checking and removing dead or dying individuals (Mortality). • Renew the sucrose solution if necessary.

Day 3 ***(……)*** **9h** **13h**	 **<u>Daily checks :</u>** ● Checking and removing dead or dying individuals (Mortality). ● Renew the sucrose solution if necessary. **<u>Preparation of feeding solutions</u>** ● Final treated or control solutions (dilutions).
Day 4 ***(……)*** **8h** **8h30** **9h00** **13h**	 **<u>Checking and removing dead or dying individuals</u>** **<u>Exposure of individuals</u>** ● Anesthetize the females. ● Weigh the females individually. ● Place 2µL of treated solution on their thorax. **<u>Wait for bees to wake up</u>** ● Place the females in individual cages. **<u>Check</u>** ● Mortality. ● Sublethal effects.
Day 4 ***(……)*** ***Day 5*** ***(……)*** ***Day 6*** ***(……)*** ***Day 7*** ***(……)***	**<u>Check</u>** ● Mortality. ● Sublethal effects.

3.2. Social bee species

Schedule	***Protocol***
Day 1 ***(……)***	**<u>Preparation of sucrose solution</u>** **<u>Reception and storage of colonies</u>** ● Place the colonies in a room at 20°C and 65% RH. ● Feed them with sucrose *ad libitum* and pollen candy.

Days 3, 5 (……)	
9h	**<u>Daily checks :</u>** ● Add a pollen candy. ● Renew the sucrose solution if necessary.
Day 7 (……)	
9h	**<u>Storage of females</u>** ● Place the females in a room at 20°C and 65% RH (10 females per box). ● Feed them with sucrose *ad libitum*.
13h	**<u>Preparation of feeding solutions</u>** ● Final treated or control solutions (dilutions).
Day 8 (……)	
8h	**<u>Checking and removing dead or dying individuals</u>**
8h30	**<u>Exposure of individuals</u>** ● Anesthetize the females. ● Weigh the females individually. ● Place 2µL of treated solution on their thorax.
9h	**<u>Wait for bees to wake up</u>** ● Place the females in individual cages.
13h	**<u>Check</u>** ● Mortality. ● Sublethal effects.
Day 9 (……) ***Day 10 (……)*** ***Day 11 (……)*** ***Day 12 (……)***	**<u>Check</u>** ● Mortality. ● Sublethal effects. **<u>Daily checks :</u>** ● Checking and removing dead or dying individuals (Mortality). ● Renew the sucrose solution if necessary.

Protocol for assessing pesticide toxicity in the lab on bee larvae

1. CONTEXT

1.1. Aim of the experiment

The aim of this experiment is to orally expose bees larvae acutely to two insecticides (Acetamiprid, Cypermethrin) and a fungicide (Tebuconazole), separately and in combination, to measure the acute toxicity of these products. It is based on Kato *& al*. (2022) ; Barraud *& al*. (2022) and Dharampal *& al*. (2018).

It can be adapted for males.

1.2. General overview

On day 1 of the study, larvae (Table 1) are placed individually in culture plates and fed a standardized amount of artificial diet. When the larvae reach the L3 stage, a single dose of the chemical tested is administered orally. Mortality is recorded up to 72 hours after the test.

Table 1. Selection of bee species:

Species	Laboratory	Females	Males	Period
Bombus terrestris	Mons	Colonies Jean Habay (breeder)	Microcolonies	2024
Bombus hypnorum	Mons	Colonies Jean Habay (breeder)	Microcolonies	2024
Bombus pascuorum	Mons	Colonies Jean Habay (breeder)	Microcolonies	2024
Bombus lapidarius	Mons	Colonies Jean Habay (breeder)	Microcolonies	2024
Anthophora plumipes	Halle	Cocoons from nest boxes	Cocoons from nest boxes	June-August 2024

2. METHODS

2.1. Material preparation

Material	Check box
Incubators	
24-well (*Bombus* sp) and 48 (solitary species) culture plates	
Humidity chamber (*Bombus sp*): 1) Box (20x20x8.8) 2) Paper towel 3) Saturated NaCl solution	
Glass micropipettes (for *Bombus sp*)	
Feeding solution (*Bombus sp*): 1) Sucrose 2) water 3) Heather pollen 4) Yeast extract 5) Sodium caseinate 6) Methanol 7) Pesticides (? g/mol)	
Pollen (adapted to the species)	
Heating plates	
Balance	
Syringe and antibacterial filter	
Flat nose pliers	
Dissecting tools	

2.2. Food preparation

Table 2. Methods to feed bee larvae:

Species	Food
Bombus terrestris	Feeding solution with : ● 5mL of sucrose solution (50%), ● 2.5mL pollen solution (40%), ● 1mL yeast extract solution (10%), ● 1mL sodium caseinate solution (1%), ● 0.5mL autoclaved mineral water.
Bombus hypnorum	Feeding solution with : ● 5mL of sucrose solution (50%), ● 2.5mL pollen solution (40%), ● 1mL yeast extract solution (10%), ● 1mL sodium caseinate solution (1%), ● 0.5mL autoclaved mineral water.
Bombus pascuorum	Feeding solution with : ● 5mL of sucrose solution (50%), ● 2.5mL pollen solution (40%), ● 1mL yeast extract solution (10%), ● 1mL sodium caseinate solution (1%), ● 0.5mL autoclaved mineral water.
Bombus lapidarius	Feeding solution with : ● 5mL of sucrose solution (50%), ● 2.5mL pollen solution (40%), ● 1mL yeast extract solution (10%), ● 1mL sodium caseinate solution (1%), ● 0.5mL autoclaved mineral water.
Osmia bicornis	Pollen candy : ● Pollen : ● Mixed with syrup (33%)
Osmia brevicornis	Pollen candy : ● Pollen : ● Mixed with syrup (33%)
Anthophora plumipes	Pollen candy : ● Pollen : ● Mixed with syrup

Concerning *Bombus* species :

1. Place 5g of sodium caseinate in 50mL of mineral water and 20g of yeast extract in 20mL of mineral water in glass vials and autoclave the solutions.
2. Add 50g of sucrose and 64g of pollen to 50mL and 40mL of autoclaved mineral water respectively. Store these solutions in a cool place at 4°C and cover with aluminium foil. Finally, expose the paper towels and spatulas to UV light.
3. Prepare 10mL of a feeding solution containing:

- 5mL of filtered sucrose solution (50%),
- 2.5mL pollen solution (40%),
- 1mL yeast extract solution (10%),
- 1mL sodium caseinate solution (1%),
- 0.5mL autoclaved mineral water.

4. Make 1.5L of saturated sodium chloride solution (1500mL distilled water and 500g NaCl) to ensure humidity in the wet chambers.

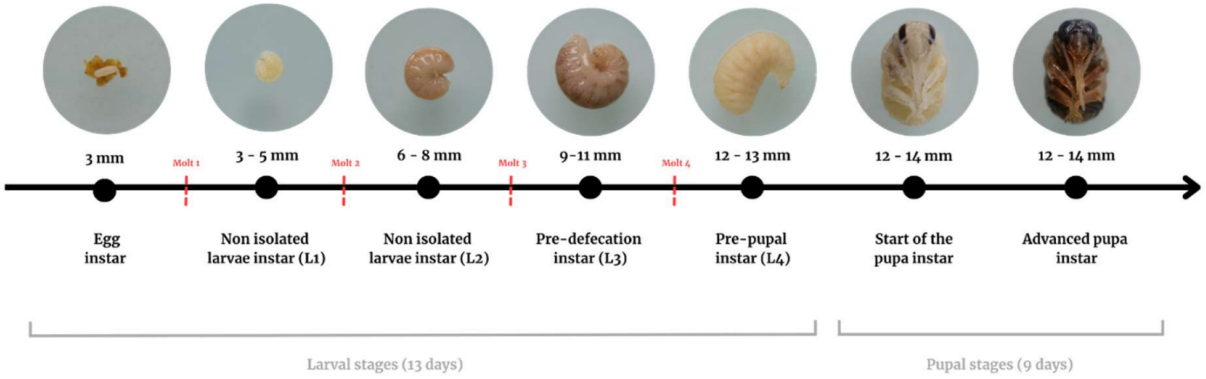
Concerning solitary bee species:

1. Fill the cells of plates with an adapted amount of pollen candy (pollen mixed with syrup).

2.3. Reception species and acclimatization

Concerning *Bombus* species:

Example of development cycle of B. terrestris workers



1. After 1 week of colony acclimatization, preheat the incubator to the adapted temperature.
2. Prepare 8 wet chambers by placing 10 sheets of paper towel soaked in saturated sodium solution at the bottom of the chambers.
3. Clean the hood, incubator, dissecting tools, hot plates and petri dishes with a bleach solution.
4. Under red light in the rearing room, remove the adults from the colonies and place them in a replacement colony.
5. Preheat the hot plates to the adapted temperature in a hood and place the culture plates on them.
6. Using dissecting forceps, remove the cells of interest from the brood and place them in a petri dish.
7. Use flat forceps to remove the larvae.
8. Randomly distribute the larvae between the 24-well plates, placing one larva per well.
9. Place 2 plates per wet chamber in the incubator in the dark.
10. Keep the plates closed to prevent contamination and to maintain the recommended temperature and humidity.
11. From the moment of installation, deprive the larvae of food for 1 hour.

12. After 1h, feed the larvae and daily check the mortality (if no movement the larva is considered dead).

Concerning solitary bee species:

1. Use a fine brush to pick the egg/larvae and place them onto pollen provision.
2. Place 1 egg/larvae per cell.
3. Put the plates into an incubator under adapted controlled conditions.
4. Observe well plates daily under a dissecting microscope without removing the lid of the well plates. If there is no movement the larva is considered dead.

Table 3. Conditions of development of bees larvae:

Species	Storage	Conditions
Bombus terrestris	24 well plates	● 35°C ● 70-80% RH ● Constant dark
Bombus hypnorum	24 well plates	● °C (see with Jean Habay) ● RH ● Constant dark
Bombus pascuorum	24 well plates	● °C (see with Jean Habay) ● RH ● Constant dark
Bombus lapidarius	24 well plates	● °C (see with Jean Habay) ● RH ● Constant dark
Osmia bicornis	48 well plates	● 23°C ● 60% RH ● Constant dark
Osmia brevicornis	48 well plates	● 23°C ● 60% RH ● Constant dark
Anthophora plumipes	?	● °C ● RH ● Constant dark

2.4. Feeding

Concerning *Bombus* species:

1. Warm the feeding solution to 35°C by placing them in the incubator for around ten minutes.
2. Feed each larva 3 times a day (8am, 1pm, 6pm) with the feeding solution, using a glass micropipette:
    - L2 = 4µL.
    - L3 = 6µL.
3. Place the droplet on the ventral part of the larva.
4. Check the survival of the individuals during feeding.
5. To maintain the humidity level (75%), re-humidify the paper towel every 2 days using the NaCl solution.

2.5. Preparation of treated food

Treatments (in order of priority):

- 1 treatment P: positive control dimethoate.
- 1 treatment N: negative control solvent (acetone).
- 1 treatment A: Acetamiprid.
- 1 treatment B: Cypermethrin.
- 1 treatment C: Tebuconazole.
- 1 treatment D: Acetamiprid + Cypermethrin.
- 1 treatment E: Acetamiprid + Tebuconazole.
- 1 treatment F: Cypermethrin + Tebuconazole.
- 1 treatment G: Fungicide - Herbicide - Insecticide.
- 1 treatment H field concentration (extreme value).

Use acetone as a solvent for all the pesticides.

Prepare stock treatment solutions:
=> See choice of pesticides.
=> See LD50.

Store at least one aliquot of each of the feeding solutions at -20°C. Also keep the first and last steps of the dilution to validate the dosage.

2.6. Pesticide exposure

Concerning *Bombus* species:

During the transition to the L3 stage (after molting), expose the individuals to 6µL of treated solution on their abdomen.

Concerning solitary bee species:

1. Make a centrally placed depression within a new pollen candy.
2. Add the treated solution into the depression.
3. Remove the control pollen candy (for the acclimatization) and replace it with the treated candy.
4. Once the exposure period is over, replace the control pollen candy in the cell.

2.7. Measurements

1. Mortality checks at: 4/5h; 24h; 48h; 72h.
2. Mass.
3. L2-> L3 and L3 -> L4

2.8. Raw data

Data :

1. ID = Identification.
    - Treatment = O; N; P; A; B; C; D; E; F; G.
    - Stage = Adult (A) ; Larva (L).
    - Sex = Female (F) ; Male (M).
    - Individual number = 01 ; 02 ; …
    - Collection site / colony
    - Species = Test species: *Bombus terrestris* (BT); *Bombus hypnorum* (BH); *Bombus lapidarius* (BL); *Bombus pascuorum* (BP); *Anthophora plumipes* (AP); *Osmia brevicornis* (OBR); *Osmia bicornis* (OBI).
2. Treatment = O; N; P; A ; B ; C ; D ; E ; F ; G.
3. Body mass = Body mass of the individual at the start of the experiment (in g).
4. Date of exposure.
5. Hour of death.
6. Mortality = Alive (0) ; Dead (1).
7. Development time L2 to L3 (in days).
8. Development time L3 to L4 (in days).
9. Day of molt.
10. Removed before treatment (if it is the case) = Yes (1) ; No (0).

3. CHECKLIST

3.1. Social bee species

Schedule	*Protocol*	×
Day 1 ***(……)***	**Reception and storage of mother colonies (6)** ● Place the colonies in a room at room temperature. ● Feed the mother colonies with Heather pollen and syrup *ad libitum* 1 to 2 times a week. **Before starting the experiment** ● Take 5 workers at random from each colony. ● Examine their midgut under a microscope to confirm the absence of parasites. **Preparation of some of the solutions** ● Prepare the feeding solution and its aliquots. ● Prepare the treated aliquots.	
Day 6/7 ***(……)*** **9h** **10h** **11h** **13h**	**Transferring adults from the colony** ● In the breeding room, remove the adults from the colony. ● Place them in a new replacement colony. **Preparation of culture plates** ● Place the culture plates in a fume hood. ● Line the bottom of the wells with wax. **Preparation of wet chambers** ● Prepare a saturated solution of sodium chloride. ● Line the bottom of the wet chamber with kitchen paper. ● Soak the paper towel in the saturated solution. **Grafting larvae** ● Open the cells in the hood. ● Remove the larvae using flat forceps or a brush. ● Place 1 larva per well in 24-well plates. ● Place the plates in humidity chambers in an incubator at 34°C. **Feed the larvae** ● Remove the larvae from the incubator. ● Remove one plate from the humidity chamber and place it on the hot plates (35°C) in the fume hood. ● Feed the larvae by placing droplets (larval stage x 2µL) of the control solution on their abdomen using a glass micropipette.	

Days 8,9,10,11,12	**<u>30 min before feeding</u>** ● Heat the feeding solution and hotplates to 35°C.	
8h - 13h – 18h	**<u>Feeding the larvae</u>** ● Remove the larvae from the incubator. ● Remove a plate from the humidity chamber and place it on the hot plates (35°C) in the fume hood. ● Feed the larvae by placing droplets (larval stage x 2µL) of the control solution on their abdomen using a glass micropipette. ● Check for the presence of molts. ● Check for mortality.	
L3 MOLT	**<u>Exposure to pesticides</u>** ● When feeding, feed the workers with 6µL of the treated solution.	

LEPIDOPTERA

Protocol for assessing pesticide topical toxicity in the lab on butterfly caterpillars

1. CONTEXT

1.1. Aim of the experiment

This experiment aims to expose wild butterflies larvae topically and acutely to active ingredients of two insecticides (Acetamiprid and Cypermethrin) and a fungicide (Tebuconazole), separately and in combination, to measure the toxicity of these products. This protocol is based on Hoang *et al.* (2010).

1.2. General overview

Caterpillars are exposed to a treated or control solution on the top of their thorax and are fed with an untreated plant adapted to the species. Mortality is recorded daily at 4h, 24 h, 48 h, 72 h and 96h.

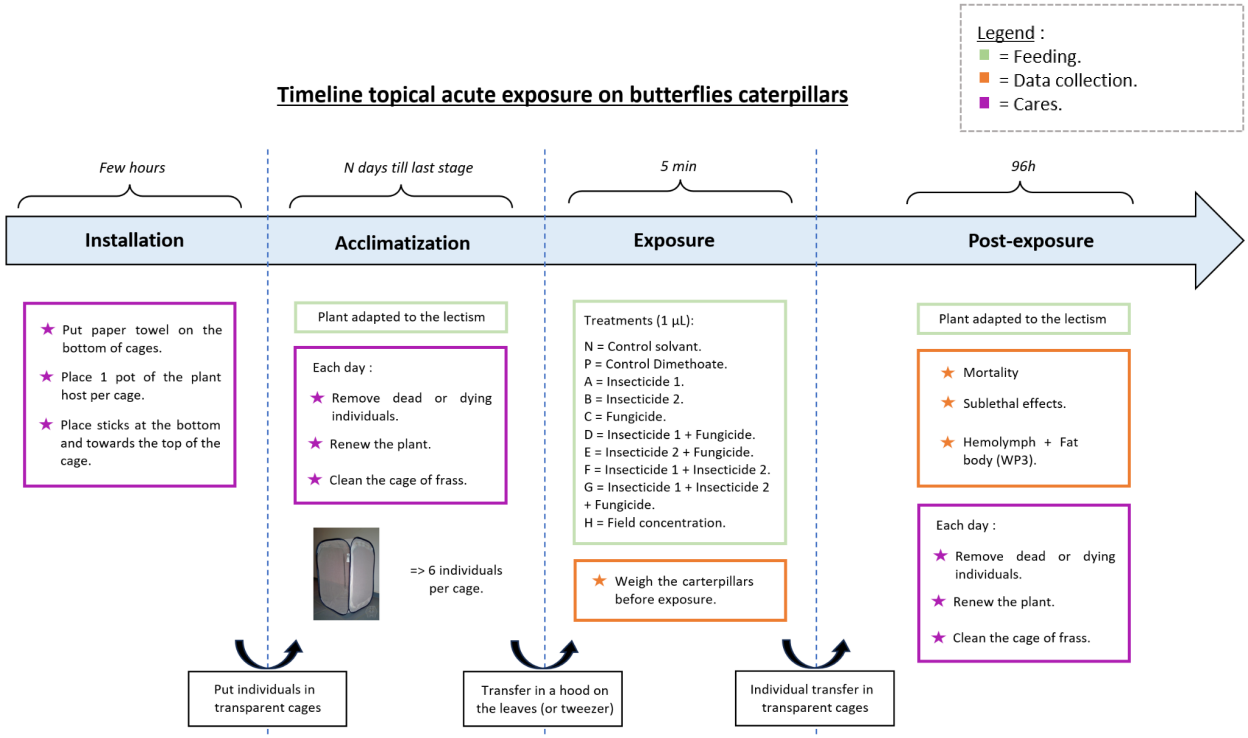


Table 1. Selection of Lepidoptera species:

Species	Laboratory	Breeder	Period
Saturnia pavonia	Mons	Jean-Pierre Vesco (France)	April/May (2024)
Aglais urticae			March/April (2024)
Papilio machaon			July (2024)
Macroglossum stellatarum			July (2024)
Maniolia jurtina			March (2025)

2. METHODS

2.1. Material preparation

Material	x
Tested species	
Tweezers for handling tested species	
Precision balance	
40 medium transparent cages	
Transparent vials	
Plant adapted to the lectism	
10 micro-applicator	
Paper towel	
Active ingredients (provided by Umons if needed)	
Solvent (acetone)	
Dimethoate	

2.2. Reception of species and acclimatization

1. Obtain L3 larvae of the selected species from the breeder.
2. Weigh the caterpillars individually in transparent vials.
3. Line the bottom of the cages with paper to make cleaning easier.

4. In each cage, place 1 pot containing 1 plant of the host plant. In addition, place a few sticks (at the bottom of the cage and towards the top) in the cage so that individuals can climb back onto the plant if one falls.
5. Transfer the caterpillars at random in the cages. Keep them in medium transparent cages (35x35x60cm: 26.101 - Cage d'élevage 35x35x60 cm - ENTO SPHINX s.r.o.) with 10 caterpillars per cage (10 treatments and 40 individuals per treatment: aim at having a minimum of n=30 individuals per treatment). Maintain individuals under laboratory conditions as described in the table below.
6. Store them till they reach the last caterpillar instar.
7. During acclimatization, check for the presence of dead or sick individuals.
8. Renew the plant when consumed or dried out and clean their containers each 3 days maximum to limit frass.

Table 2. Methods for maintaining caterpillars under controlled conditions:

Species	Conditions	Food
Saturnia pavonia	• 20°C • 50-70% • Natural light	• *Rubus fruticosus.*
Aglais urticae	• 24°C • 40-60% • Natural light	• *Urtica dioica*.
Papilio machaon	• 24°C • 50-70% RH • 15:9	• *Foeniculum vulgare.*
Macroglossum stellatarum	• 25°C • 40-50% • Natural light	• *Galium aparine.*
Maniolia jurtina	• 25°C • 50-60% • Natural light	• *Phalaris arundinacea.* • OR *Arrhenatherum elatius.*

2.3. Preparation of solutions

Treatments (in order of priority):

- 1 treatment P: positive control dimethoate.
- 1 treatment N: negative control solvent (acetone).
- 1 treatment A: Acetamiprid.
- 1 treatment B: Cypermethrin.
- 1 treatment C: Tebuconazole.
- 1 treatment D: Acetamiprid + Cypermethrin.
- 1 treatment E: Acetamiprid + Tebuconazole.
- 1 treatment F: Cypermethrin + Tebuconazole.
- 1 treatment G: Fungicide - Herbicide - Insecticide.
- 1 treatment H field concentration (extreme value).

Use acetone as a solvent for all the pesticides.

Prepare stock treatment solutions:
=> See choice of pesticides.
=> See LD50 of *Bombus terrestris* and adjust according to the weight of the caterpillars.

1. Calculate the mean body mass of the test species based on the weighing performed.
2. Adjust the *B.terrestris* LD50 by multiplying it with the ratio of the mean body mass of the test species and the *B.terrestris* body mass, *i.e.*, LD50-*B.terrestris** (mean body mass test species/ mean body mass *B.terrestris*).

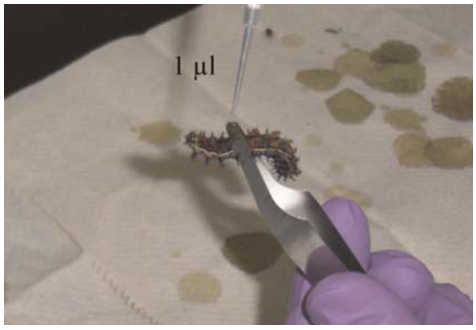
Store at least one aliquot of each of the feeding solutions at -20°C. Also keep the first and last steps of the dilution to validate the dosage.

2.4. Pesticide exposure

1. Transfer the caterpillar under the hood thanks to a leaf / stick or use a tweezer when possible (tests have to be made with some larvae before the start of the experiment).
2. Expose individuals to 1 µL (adapt it to the mean mass of the species) of the treatment on their thorax (between the 2 last thoracic segments).
3. Allow the solution to air dry for 15 sec.

4. After exposure, place them in individual cages.

5. Observe individuals for 96 h. They are considered alive if they move or respond to stimulation.



2.5. Measurements

1. Mortality checks at: 4h; 24h; 48h; 72h and 96h.
2. Mass.
3. Sublethal effects: unusual behavior; paralysis; reduced coordination.

For WP3, collect haemolymph and fat bodies for proteome studies and for transcriptome studies, preferably from the same specimens, using specimens living at 48 hrs post treatment. Details are to be provided in protocols from WP3. Collect a minimum of n=3 haemolymph samples per treatment and n = 8 fat body samples per treatment (do not use the same individuals for the haemolymph and fat body samples as a stressful haemolymph sampling will change the transcriptome).The most sensitive species will initially be studied.

2.6. Raw data

ID	Treat-ment	Mass	Mortalit_at_4h	Mortality_at_24h	Mortality_at_48h	Hour_of_death
LF01SP	N	1.2	0	1	1	24
LF02AU	A	1.1	0	0	0	0

ID	Sublethal _effects	Description_of_sublethal _effects	Removed_before _treatment	Cage_allocation
LF01SP	1	Paralysis	0	1
LF02AU	0	None	0	3

Data:

1. ID = Identification.
    - Stage = L (Larvae) ; A (Adults).
    - Treatment = O; N; P; A; B; C; D; E; F; G.
    - Individual number = 01 ; 02 ; …
    - Species : *Aglais urticae* (AU) ; *Saturnia pavonia* (SP) ; *Macroglossum stellatarum* (MS) ; *Papilio machaon* (PM) ; *Maniola jurtina* (MJ).
2. Treatment = O; N; P; A ; B ; C ; D ; E ; F ; G.
3. Body mass = Body mass of the individual at the start of the experiment (in g).
4. Hour of death.
5. Mortality = Alive (0) ; Dead (1).
6. Sublethal effects = Yes (1) ; No (0).
7. Description of sublethal effects.
8. Remove before treatment (if it is the case) = Yes (1) ; No (0).
9. Cage allocation = Cage number of the treatment.

3. CHECKLIST

Schedule	***Protocol***	**×**
Day 1 ***(……)***	**Reception and storage of caterpillars** ● Weigh the individuals in vials. ● Place at random the individuals in cages. ● Feed them with their respective host plant.	
Days …….. ***(……)*** ***(……)*** ***(……)*** ***(……)*** ***(……)*** ***(……)***	**Daily check (9h)** ● Remove dead or sick individuals. ● Renew the plant every day. ● Clean the cage of frass.	

Day …. ***(…….)***	**<u>Exposure of individuals (10h)</u>** ● Carefully take the larva between tweezers or leaf. ● Place 1µL of treated solution on their thorax. ● Allows it to dry for 15 sec. ● Place the caterpillars in individual cages. **<u>Check (14h)</u>** ● Mortality. ● Sublethal effects.	
Days …….. ***(…….)*** ***(…….)*** ***(…….)*** ***(…….)***	**<u>Daily check (10h)</u>** ● Mortality (Remove dead or sick individuals). ● Renew the plant. ● Clean the cage of frass. ● Sublethal effects.	

Protocol for assessing the topical toxicity of pesticides on wild butterfly adults

1. CONTEXT

1.1. Aim of the experiment

This experiment aims to expose wild butterfly larvae topically and acutely to active ingredients of two insecticides (Acetamiprid and Cypermethrin) and a fungicide (Tebuconazole), separately and in combination, to measure the toxicity of these products. This protocol is based on Hoang *& al.* (2010).

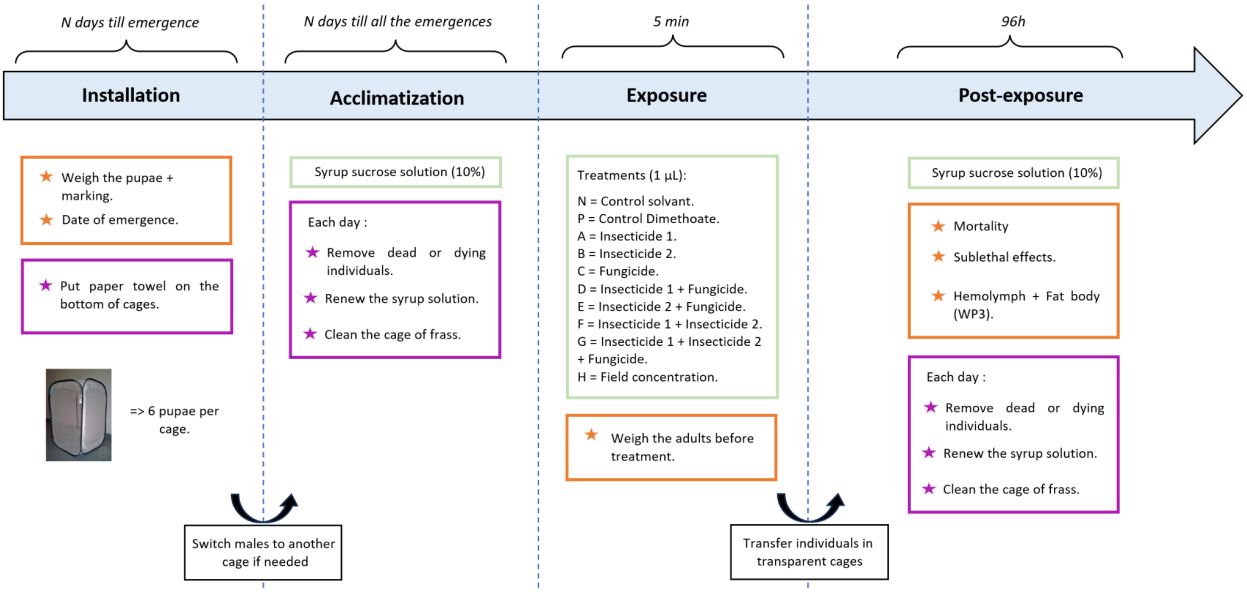
1.2. General overview

Butterflies are exposed to a treated or control solution on the top of their thorax and are fed with an untreated syrup (except *Saturnia pavonia* that do not feed in the imago stage). Mortality is recorded daily at 4h, 24 h, 48 h, 72h and 96h.

Table 1. Selection of Lepidoptera species:

Species	Laboratory	Breeder	Period
Saturnia pavonia	Mons	Jean-Pierre Vesco (France)	April/May (2025)
Aglais urticae			March/April (2024)
Papilio machaon			July (2025)
Macroglossum stellatarum			July (2024)
Maniolia jurtina			March (2026)

Timeline topical acute exposure on adults butterflies



Legend :
- = Feeding.
- = Data collection.
- = Cares.

2. METHODS

2.1. Material preparation

Material	x
Tested species	
Tweezers for handling tested species	
Precision balance	
40 large transparent cages	
Glassine envelops	
Plant adapted to the lectism	
Artificial petals	
80 metal wires	
400 points of a P20 or P200 pipette tip sealed	
400 Eppendorfs	
10 micro-applicators	
Paper towel	
Pesticides	
Solvent (acetone)	
Dimethoate	

2.2. Reception of species and acclimatization before the emergence

1. Obtain pupae of the selected species from the breeder.
2. Weigh the pupae.
3. Line the bottom of the cages with paper to make cleaning easier.
4. Set the pupae at random in the cages. Keep them in large transparent cages (60x60x60cm: 26.102 - Cage d'élevage 60x60x90 cm - ENTO SPHINX s.r.o.) with 10 pupae per cage (10 treatments and 40 individuals per treatment: aim at having a minimum of n=30 individuals per treatment). Maintain individuals under laboratory conditions as described in the table 2 below. Use different cages per sex if there is pupal sexual dimorphism in the species (or after the emergence if there is imago sexual dimorphism).
5. Store them till the emergence.

Table 2. Methods for maintaining caterpillars under controlled conditions:

Species	Conditions	Food
Saturnia pavonia	• 20°C • 50-70% • Natural light	• Does not eat as imago.
Aglais urticae	• 24°C • 40-60% • Natural light	• 10% sucrose solution.
Papilio machaon	• 24°C • 50-70% RH • 15:9	• 10% sucrose solution.
Macroglossum stellatarum	• 25°C • 40-50% • Natural light	• 10% sucrose solution.
Maniolia jurtina	• 25°C • 50-60% • Natural light	• 10% sucrose solution.

2.3. Acclimatization after emergence

1. When the imagos start to emerge, add artificial flowers (filled with a 10% solution of sucrose and mineral water *i.e.,* sucrose solution) attached to the bottom and the top of the cage thanks to the metal wires. Add 2 of these structures per cage.
2. If needed, train the butterflies to eat by carefully handling them and dipping their proboscis into the syrup solution.
3. Weigh emergents by using glassine envelopes. Mark them to follow the emergences.

4. During acclimatization, check for the presence of dead or sick individuals and remove them. Renew the syrup solutions every day and clean their containers each day to limit frass.

2.4. Preparation of solutions

Treatments (in order of priority):

- 1 treatment P: positive control dimethoate.
- 1 treatment N: negative control solvent (acetone).
- 1 treatment A: Acetamiprid.
- 1 treatment B: Cypermethrin.
- 1 treatment C: Tebuconazole.
- 1 treatment D: Acetamiprid + Cypermethrin.
- 1 treatment E: Acetamiprid + Tebuconazole.
- 1 treatment F: Cypermethrin + Tebuconazole.
- 1 treatment G: Fungicide - Herbicide - Insecticide.
- 1 treatment H field concentration (extreme value).

Use acetone as a solvent for all the pesticides.

Prepare stock treatment solutions:
=> See choice of pesticides.
=> See LD50 of *Bombus terrestris* and adjust according to the weight of the caterpillars.

3. Calculate the mean body mass of the test species based on the weighing performed.
4. Adjust the *B.terrestris* LD50 by multiplying it with the ratio of the mean body mass of the test species and the *A.urticae* body mass, *i.e.*, LD50-*B.terrestris** (mean body mass test species/ mean body mass *B.terrestris*).

Store at least one aliquot of each of the feeding solutions at -20°C. Also keep the first and last steps of the dilution to validate the dosage.

2.5. Pesticide exposure

1. Transfer the caterpillar under the hood thanks to a leaf / stick or use a tweezer when possible (tests have to be made with some larvae before the start of the experiment).
2. Expose individuals to 1 µL (adapt it to the mean mass of the species) of the treatment on their thorax.
3. Allow the solution to air dry for 15 sec.

4. After exposure, replace them in the cage.
5. Observe individuals for 96 h. They are considered alive if they move or respond to stimulation.

2.6. Measurements

1. Mortality checks at: 4h; 24h; 48h; 72h and 96h.
2. Mass.
3. Sublethal effects: unusual behavior; paralysis; reduced coordination.

For WP3, collect haemolymph and fat bodies for proteome studies and for transcriptome studies, preferably from the same specimens, using specimens living at 48 hrs post treatment. Details are to be provided in protocols from WP3. Collect a minimum of n=3 haemolymph samples per treatment and n = 8 fat body samples per treatment (do not use the same individuals for the haemolymph and fat body samples as a stressful haemolymph sampling will change the transcriptome). The most sensitive species will initially be studied.

2.7. Raw data

ID	Treat-ment	Sex	Mass	Mortalit_at_4h	Mortality_at_24h	Mortality_at_48h	Hour_of_death
AF01SP	N	F	1.2	0	1	1	24
AF02AU	A	F	1.1	0	0	0	0

ID	Sublethal _effects	Description_of_sublethal _effects	Removed_before _treatment	Cage_allocation
AF01SP	1	Paralysis	0	1
AF02AU	0	None	0	3

Data:

1. ID = Identification.
    - Stage = L (Larvae) ; A (Adults).
    - Treatment = O; N; P; A; B; C; D; E; F; G.
    - Individual number = 01; 02; …
    - Species : *Aglais urticae* (AU) ; *Saturnia pavonia* (SP) ; *Macroglossum stellatarum* (MS) ; *Papilio machaon* (PM) ; *Maniola jurtina* (MJ).
2. Treatment = O; N; P; A; B; C; D; E; F; G.
3. Sex = Female (F) ; Male (M).
4. Body mass = Body mass of the individual at the start of the experiment (in g).
5. Hour of death.
6. Mortality = Alive (0) ; Dead (1).
7. Sublethal effects = Yes (1) ; No (0).
8. Description of sublethal effects.
9. Remove before treatment (if it is the case) = Yes (1) ; No (0).
10. Cage allocation = Cage number of the treatment.

3. CHECKLIST

Schedule	*Protocol*	×
Day 1 ***(……)***	**Reception and storage of pupae** ● Weigh the individuals. ● Place at random the pupae in cages.	
Days …… ***(……)*** ***(……)*** ***(……)***	**Daily check (8h)** ● Emergent (mark it and weigh it). ● Renew the syrup solution every day. ● Clean the cage of frass. **Daily check (13h and 18h)** ● Emergent (mark it).	
Day …. ***(……)***	**Exposure of individuals (10h)** ● Take the wings of the adult. ● Place 1µL of treated solution on their thorax. ● Allow it to dry for 15 sec. ● Replace the imago in the cage. ● Renew the syrup solution and clean the cage. **Check (14h)** ● Mortality. ● Sublethal effects.	
Days …… ***(……)*** ***(……)*** ***(……)*** ***(……)***	**Daily check (10h)** ● Mortality (Remove dead or sick individuals). ● Renew the syrup solution. ● Clean the cage of frass. ● Sublethal effects.	

Protocol for assessing the oral toxicity of pesticides on wild butterfly adults

1. CONTEXT

1.1. Aim of the experiment

This experiment aims to expose wild butterfly larvae orally and acutely to active ingredients of two insecticides (Acetamiprid and Cypermethrin) and a fungicide (Tebuconazole), separately and in combination, to measure the toxicity of these products.

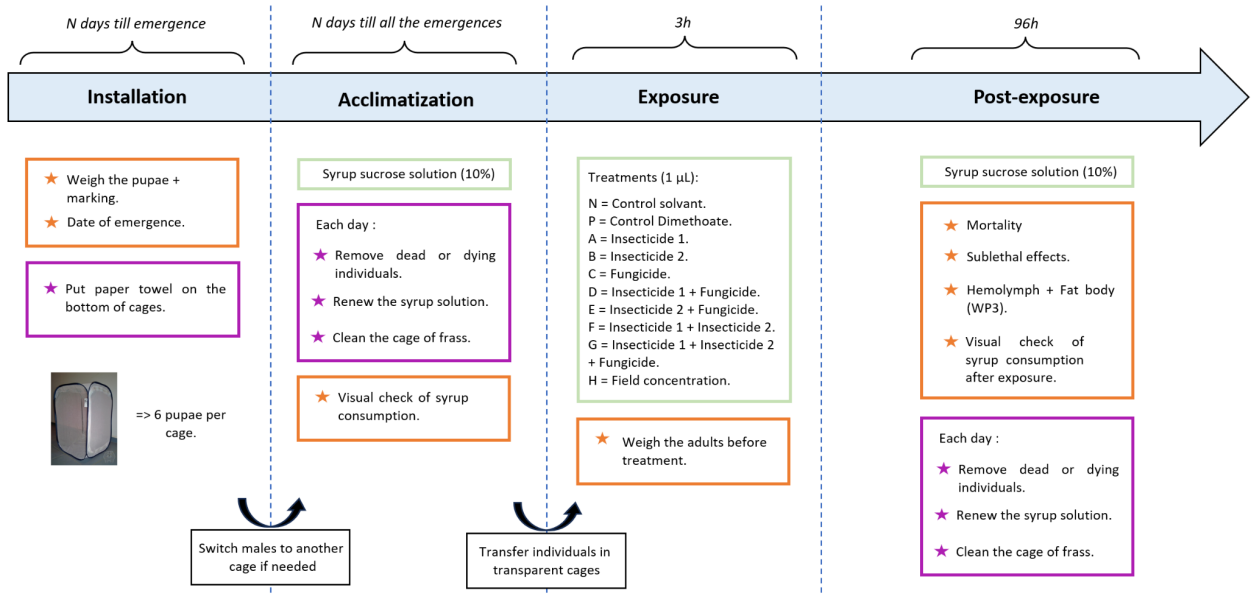
1.2. General overview

Butterflies are fed with a sucrose control solution or a treated solution. Mortality is recorded daily at 4h, 24 h, 48 h, 72h and 96h.

Table 1. Selection of lepidoptera species:

Species	Laboratory	Breeder	Period
Saturnia pavonia	Mons	Jean-Pierre Vesco (France)	April/May (2025)
Aglais urticae			March/April (2024)

Timeline oral acute exposure on adults butterflies



Legend :
- = Feeding.
- = Data collection.
- = Cares.

2. METHODS

2.1. Material preparation

Material	x
Test species	
Tweezers for handling test species	
Precision balance	
Large transparent cages	
Glassine envelops	
Plant adapted to the lectism	
Petals made from tape	
40 metal wire	
400 points of a P20 or P200 pipette tip sealed	
400 Eppendorf tubes	
10 micro-applicator	
Paper towel	
Pesticides (provided by Umons)	
Solvent (acetone)	
Dimethoate	

2.2. Reception of species and acclimatization before the emergence

1. Obtain pupae of the selected species from the breeder.
2. Weigh the pupae.
3. Line the bottom of the cages with paper to make cleaning easier.
4. Set the pupae at random in the cages. Keep them in large transparent cages (60x60x60cm: 26.102 - Cage d'élevage 60x60x90 cm - ENTO SPHINX s.r.o.) with 10 pupae per cage (10 treatments and 40 individuals per treatment: aim at having a minimum of n=30 individuals per treatment). Maintain individuals under laboratory conditions as described in the table 2 below.
5. Store them till the emergence.

Table 2. Methods for maintaining caterpillars under controlled conditions:

Species	Conditions	Food
Aglais urticae	• 24°C • 40-60% • Natural light	• 10% sucrose solution.
Macroglossum stellatarum	• 25°C • 40-50% • Natural light	• 10% sucrose solution.

2.3. Acclimatization after emergence

1. When the imagoes start to emerge, add artificial flowers (filled with a 10% solution of sucrose and mineral water *i.e.,* sucrose solution) attached to the bottom and the top of the cage thanks to the metal wires. Add 2 of these structures per cage. Use different cages per sex if there is imago sexual dimorphism.
2. If needed, train the butterflies to eat by carefully handling them and dipping their proboscis into the syrup solution.
3. Weigh emergents by using glassine envelopes. Mark them to follow the emergences.
4. During acclimatization, check for the presence of dead or sick individuals and remove them. Renew the syrup solutions every day and clean their containers each day to limit frass.

2.4. Preparation of solutions

Treatments (in order of priority):

- 1 treatment P : positive control dimethoate.
- 1 treatment N : negative control solvent (acetone).
- 1 treatment A : Acetamiprid.
- 1 treatment B : Cypermethrin.
- 1 treatment C : Tebuconazole.
- 1 treatment D : Acetamiprid + Cypermethrin.
- 1 treatment E : Acetamiprid + Tebuconazole.
- 1 treatment F : Cypermethrin + Tebuconazole.
- 1 treatment G : Fungicide - Herbicide - Insecticide.
- 1 treatment H field concentration (extreme value).

Use the acetone as solvent for all the pesticides.

Prepare stock treatment solutions:
=> See choice of pesticides.
=> See LD50 of *Bombus terrestris* and adjust according to the weight of the caterpillars.

1. Calculate the mean body mass of the test species based on the weighing performed.
2. Adjust the *B. terrestris* LD50 by multiplying it with the ratio of the mean body mass of the test species and the *A. urticae* body mass, *i.e.*, LD50-*B. terrestris** (mean body mass test species/ mean body mass *B. terrestris*).

Store at least one aliquot of each of the feeding solutions at -20°C. Also keep the first and last steps of the dilution to validate the dosage.

2.5. Pesticide exposure

1. Transfer the individuals in small cages (1 individual per cage).
2. Expose the individuals to the treated solution in the P200 sealed pipettes during 3h.
3. After exposure, leave the adults in the individual cage.
4. Observe individuals for 96h. They are considered alive if they moved or responded to stimulation.

2.6. Measurements

1. Mortality checks at: 4h; 24h; 48h; 72h; 96h.
2. Mass of the pupae.
3. Mass of the adult.
4. Treated solution consumption (visual check).
5. Sublethal effects: unusual behavior; paralysis; reduced coordination.

For WP3, collect haemolymph and fat bodies for proteome studies and for transcriptome studies, preferably from the same specimens, using specimens living at 48 hrs post treatment. Details are to be provided in protocols from WP3. Collect a minimum of n=3 haemolymph samples per treatment and n = 8 fat body samples per treatment (do not use the same individuals for the haemolymph and fat body samples as a stressful haemolymph sampling will change the transcriptome).The most sensitive species will initially be studied.

In case of *Aglais urticae* at each stage of mortality check, collect dead individuals separately to Eppendorf tubes marked with a specific code (Species_Mass_T_MC_XX), freeze and send to PIWet for toxicokinetic analysis.
Codes should be entered as **Species_Mass_T_MC_XX**
where:
Species – *Aglais urticae* (AU)
Mass - mass of the individual insect before pesticides exposure (in g)
T - Treatment Identifier (P, N, A, B, C, D, E, F, G or H)
MC – Mortality Check (4h, 24h, 48h, 72h or 96h)
XX – Amount of treated solution (µL)
Individuals after haemolymph collection also send to PIWet including additional mark

on the label (H): Species_Mass_T_MC_XX**_H**
Individuals after fat bodies collection will not be tested by PIWet.

2.7. Raw data

ID	Treat -ment	Sex	Mass_Pupa	Mass_I mago	Mortalit_at _4h	Mortality_at_ 24h	Mortality_at _48h	Hour_of_d eath
AF01SP	N	F	1.2	1.2	0	1	1	24
AF02AU	A	F	1.1	1.1	0	0	0	0

ID	Treated_solution_ consumption	Sublethal_ef fects	Description_of_sublethal_ effects	Removed_before _treatment	Cage_allocation
AF01SP		1	Paralysis	0	1
AF02AU		0	None	0	3

Data :

1. ID = Identification.
    - Stage = L (Larvae) ; A (Adults).
    - Treatment = O; N; P; A; B; C; D; E; F; G.
    - Individual number = 01; 02; …
    - Species : *Aglais urticae* (AU) ; *Saturnia pavonia* (SP) ; *Macroglossum stellatarum* (MS) ; *Papilio machaon* (PM) ; *Maniola jurtina* (MJ).
2. Treatment = O; N; P; A; B; C; D; E; F; G.
3. Sex = Female (F) ; Male (M).
4. Body mass of pupa = Body mass of the individual at the start of the experiment (in g).
5. Body mass of the imago =Body mass of the individual at the emergence (in g).
6. Hour of death.
7. Mortality = Alive (0) ; Dead (1).
8. Sublethal effects = Yes (1) ; No (0).
9. Description of sublethal effects.
10. Remove before treatment (if it is the case) = Yes (1) ; No (0).
11. Cage allocation = Cage number of the treatment.

3. CHECKLIST

Schedule	*Protocol*
Day 1 ***(…….)***	**Reception and storage of pupae** ● Weigh the individuals. ● Place at random the pupae in cages.
Days …….. ***(…….)*** ***(…….)*** ***(…….)***	**Daily check (9h)** ● Emergent (mark it). ● Renew the syrup solutions every day (weigh it). ● Clean the cage of frass. **Daily check (13h)** ● Emergent (mark it and weigh it).
Day …. ***(…….)***	**Exposure of individuals (10h)** ● Replace the syrup solutions by the treated solutions. ● Wait 3h. ● Weigh the treated solutions. ● Renew the syrup solutions (visual check) and clean the cage. **Check (14h)** ● Mortality. ● Sublethal effects.
Days …….. ***(…….)*** ***(…….)*** ***(…….)*** ***(…….)***	**Daily check (10h)** ● Mortality (Remove dead or sick individuals). ● Renew the syrup solutions. ● Clean the cage of frass. ● Sublethal effects.

HOVERFLIES

Protocol for assessing pesticide topical toxicity in the lab on hoverfly adults

1. CONTEXT

1.1. Aim of the experiment

This experiment aims to expose syrphid adults topically and acutely to active ingredients of two insecticides (Acetamiprid and Cypermethrin) and a fungicide (Tebuconazole), separately and in combination, to measure the toxicity of these products. This protocol is based on OECD guidelines 246 and 214.

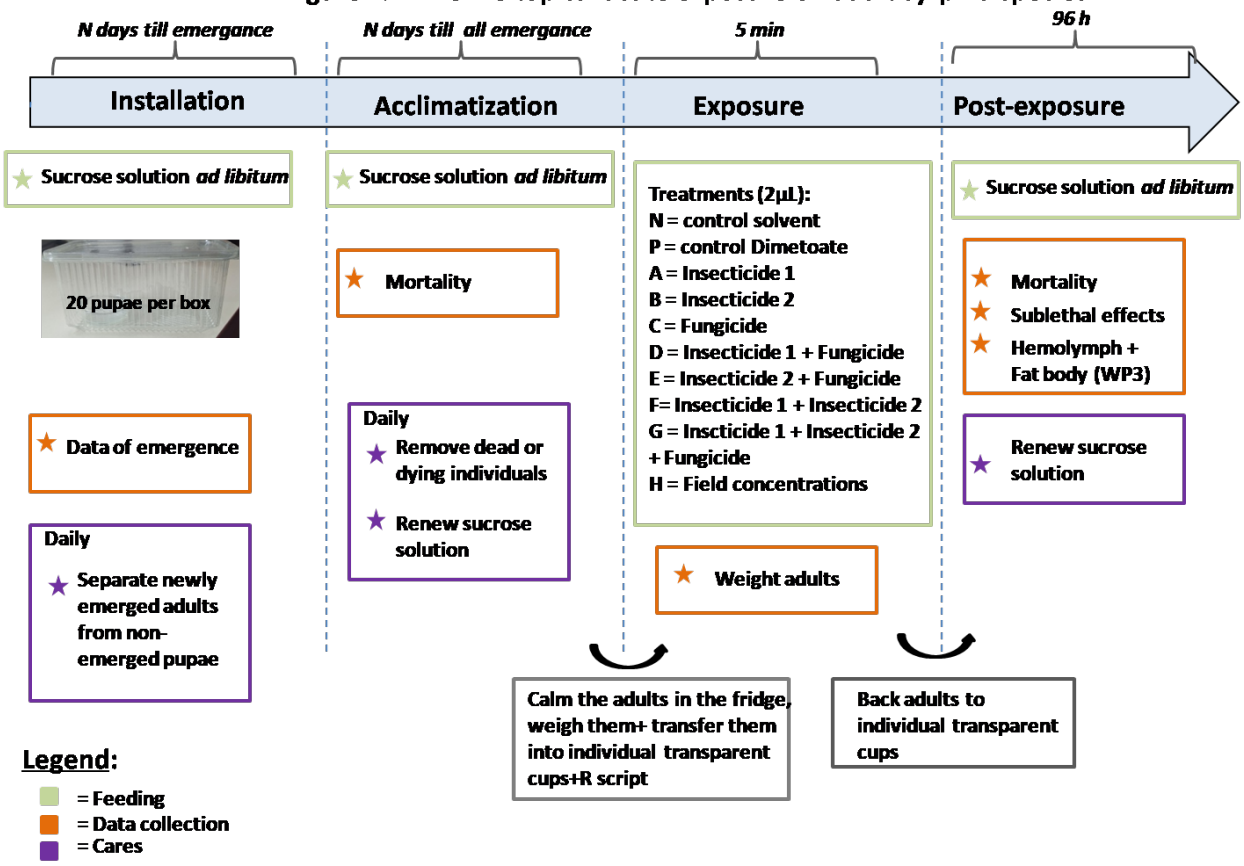
1.2. General overview

Hoverflies are exposed to a treated or control solution on the top of their thorax and are fed with untreated syrup. Mortality is recorded daily at 4h, 24 h, 48 h, 72 h and 96h (Fig 1).

Table 1. Selection of Syrphidae species:

Species	Laboratory	Breeder	Period
Eristalis arbustorum	UNSPMF	Santos Rojo, Spain	July 2024
Eristalinus aeneus		Santos Rojo, Spain	May-June 2024
Eupeodes corollae		Biobest, Belgium	March-April 2025
Myathropa florea		Santos Rojo, Spain	Jun 2024
Cheilosia canicularis		Field	Spring 2025

Figure 1. Timeline topical acute exposure on adult syrphid species



2. METHODS

2.1. Material preparation for one treatment per species

Tested species
Tweezers for handling tested species
Precision balance
6 large transparent boxes
40 small transparent boxes
40 eppendorf tube (2 mL)
Paper towel
Sucrose solution 50% w/v
Pesticides
Solvent (acetone)
Dimethoate

2.2. Reception of species and acclimatization

1. Obtain pupae of the selected species from the breeder.
2. Line the bottom of the boxes (2 L, Fig 2A) with paper to make cleaning easier.
3. Set the pupae at random in the boxes. Keep them in large transparent boxes with 20 pupae placed in a Petri dish per boxes provided with a 50% w/w sugar solution (10 treatments and 40 individuals per treatment: aim at having a minimum of n=30 individuals per treatment). Maintain individuals at RT and natural light.
4. Daily, collect fully emerged adults. Technically, the Petri dish with the non-emerged pupae will be taken out of the box, and placed in a new plastic box.
5. Emerged adults remain in boxes at room temperature and natural light until day before exposure.

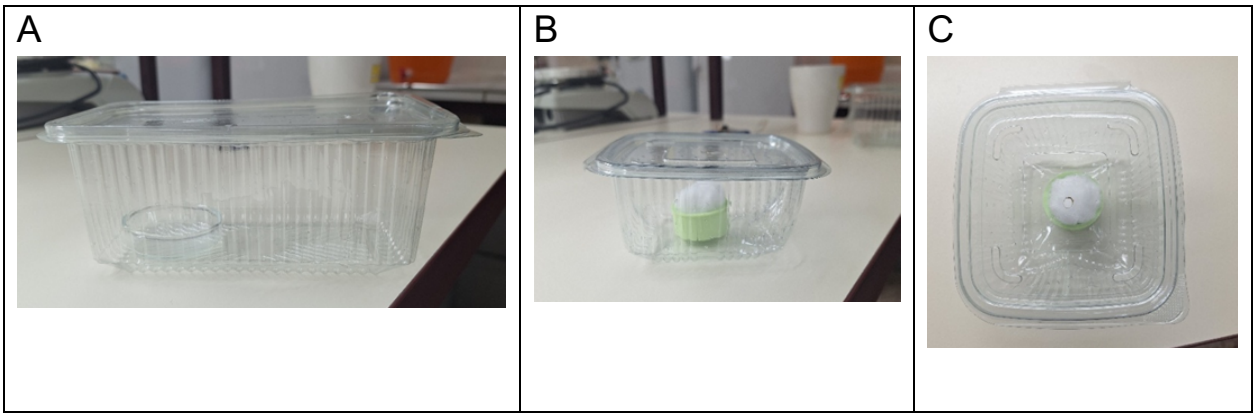


Fig. 2 – A) Transparent hoarding box; B) and C) Transparent box for individual bees

2.3. Before exposure: preparation of solutions

Treatments (in order of priority):

- 1 treatment P: positive control dimethoate.
- 1 treatment N: negative control solvent (acetone).
- 1 treatment A: Acetamiprid.
- 1 treatment B: Cypermethrin.
- 1 treatment C: Tebuconazole.
- 1 treatment D: Acetamiprid + Cypermethrin.
- 1 treatment E: Acetamiprid + Tebuconazole.
- 1 treatment F: Cypermethrin + Tebuconazole.
- 1 treatment G: Fungicide - Herbicide - Insecticide.
- 1 treatment H: field concentration (extreme value).

Use acetone as a solvent for all the pesticides.

Prepare stock treatment solutions:
=> See choice of pesticides.
=> See LD50 of *Bombus terrestris* and adjust according to the weight of the hoverfly adults.
1. Calculate the mean body mass of the test species based on the weighing performed.
2. Adjust the *B. terrestris* LD50 by multiplying it with the ratio of the mean body mass of the test species and the *B. terrestris* body mass, i.e. , LD50-*B. terrestris* * (mean body mass test species/ mean body mass *B. terrestris*).

Store at least one aliquot of each of the treatment solutions at -20°C. Also keep the first and last steps of the dilution to validate the dosage.

2.4. Pesticide exposure

Day before exposure:

1. Day before exposure, place the box with adults in the fridge until the flies calm down.
2. Take individual (one by one) flies from the fridge, weigh individual flies by placing 2mL tube on a fine balance, record the weight of the fly at a precision of at least 0.1 mg alongside the specimen ID, date of emergence and sex in the respective excel template.
3. Place the individuals in a separate transparent plastic box (Fig 2B and C) with the appropriate ID at RT and natural light until exposure.
4. According to input data in Excel doc and R script, individuals will be allocated for treatment (minimum 30 alive individuals per treatment).

On the day of exposure:

1. Place individually caged flies in the fridge to calm down. Take them one by one from the fridge and expose them to 2µL of the treatments on their thorax.
2. Allow the solution to air dry for 15 s.
3. After exposure, place back individuals in separate transparent plastic boxes with appropriate ID at RT, exposed to natural light until the end of experiment.

2.5. Measurements and observation after treatment

1. Mortality checks at: 4h; 24h; 48h; 72h and 96h.
2. Sublethal effects: unusual behavior; paralysis; reduced coordination.

For WP3, collect haemolymph and fat bodies for proteome studies and for transcriptome studies, preferably from the same specimens, using specimens living at 48 hrs post

treatment. Details are to be provided in protocols from WP3. Collect a minimum of n=3 haemolymph samples per treatment and n = 3 fat body samples per treatment.The most sensitive species will initially be studied.

2.6. Raw data

Row data will be collected in an Excel file created within the group WP2. Data includes data before exposure which will be used for allocation individuals for treatment (such as stage, sex, body mass, and created ID for every individual), and after exposure (daily check of mortality and sublethal effects).

3. CHECKLIST

Schedule	Protocol	x
Day 1	**Reception and storage of pupae** • Place at random 20 pupae per box at RT.	
Day 2 (….)	**Daily check (8h)** • Separate emerged adults from non-emerged pupae. • Transfer non-emerged pupae in a new box. • Clean the box of frass.	
Day 3 (…..)	**Exposure of individuals (10h)** • Calm down the adult one by one in the fridge. • Place 2µL of treated solution on their thorax. Allows it to dry for 15 sec. • Place back the adults in individual cups. **Check (14h)** • Mortality. • Sublethal effects.	
Days…..	**Daily check (10h)** • Mortality (Remove dead or sick individuals). • Renew the syrup solution. • Clean the box of frass. • Sublethal effects.	

Protocol for assessing pesticide oral toxicity in the lab on hoverfly adults

1. CONTEXT

1.1. Aim of the experiment

This experiment aims to expose syrphid adults orally and acutely to active ingredients of two insecticides (Acetamiprid and Cypermethrin) and a fungicide (Tebuconazole), separately and in combination, to measure the toxicity of these products. This protocol is based on OECD guidelines 247 and 213.

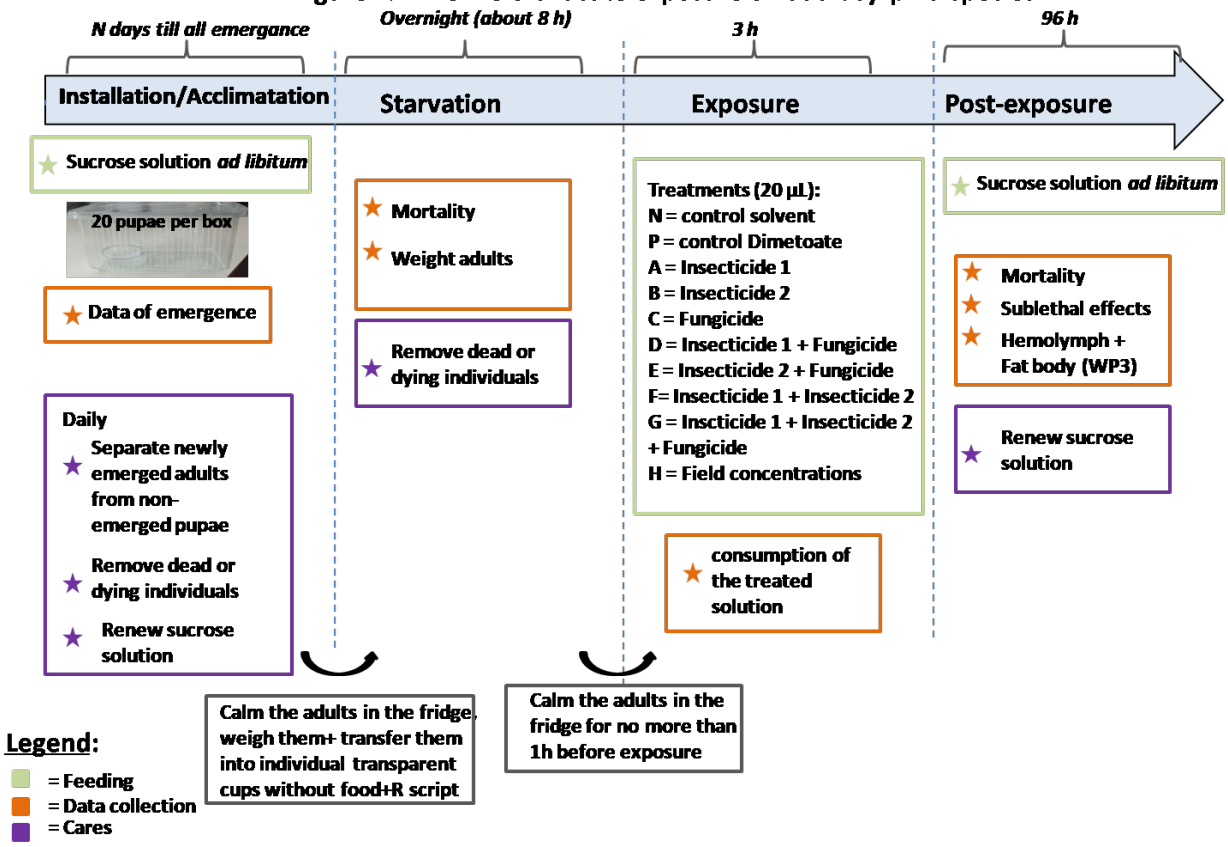
1.2. General overview

Hoverflies are fed with a sucrose control solution or a treated solution. Mortality is recorded daily at 4h, 24 h, 48 h, 72 h and 96h (Fig 1).

Table 1. Selection of Syrphidae species:

Species	Laboratory	Breeder	Period
Eristalis arbustorum	UNSPMF	Santos Rojo, Spain	October 2024
Eristalinus aeneus		Santos Rojo, Spain	November 2024
Myathropa florea		Santos Rojo, Spain	April-May 2025
Eupeodes corollae		Biobest, Belgium	March-April 2025
Cheilosia canicularis		field	Spring 2025

Figure 1. Timeline oral acute exposure on adult syrphid species



2. METHODS

2.1. Material preparation for one treatment per species

Tested species
Tweezers for handling tested species
Precision balance
6 large transparent boxes
40 small transparent boxes
40 eppendorf tube (2 mL)
Paper towel
Sucrose solution 50% w/v
Pesticides
Solvent (acetone)
Dimethoate

2.2. Reception of species and acclimatization

1. Obtain pupae of the selected species from the breeder.
2. Line the bottom of the boxes (2 L) with paper to make cleaning easier.
3. Set the pupae at random in the boxes. Keep them in large transparent boxes with 20 pupae placed in a Petri dish per boxes provided with a 50% w/w sugar solution (10 treatments and 40 individuals per treatment: aim at having a minimum of n=30 individuals per treatment). Maintain individuals at RT and natural light.
4. Daily, collect fully emerged adults. Technically, the Petri dish with the non-emerged pupae will be taken out of the box, and placed in a new plastic box.
5. Emerged adults remain in boxes at room temperature and natural light until day of starvation.

2.3. Preparation of solutions

Treatments (in order of priority):

- 1 treatment P: positive control dimethoate.
- 1 treatment N: negative control solvent (acetone).
- 1 treatment A: Acetamiprid.
- 1 treatment B: Cypermethrin.
- 1 treatment C: Tebuconazole.
- 1 treatment D: Acetamiprid + Cypermethrin.
- 1 treatment E: Acetamiprid + Tebuconazole.
- 1 treatment F: Cypermethrin + Tebuconazole.
- 1 treatment G: Fungicide - Herbicide - Insecticide.
- 1 treatment H: field concentration (extreme value).

Use acetone as a solvent for all the pesticides.
Prepare stock treatment solutions:
=> See choice of pesticides.
=> See LD50 of *Bombus terrestris* and adjust according to the weight of the hoverfly adults.
1. Calculate the mean body mass of the test species based on the weighing performed.
2. Adjust the *B. terrestris* LD50 by multiplying it with the ratio of the mean body mass of the test species and the *B. terrestris* body mass, i.e. , LD50-*B. terrestris* * (mean body mass test species/ mean body mass *B. terrestris*).

Store at least one aliquot of each of the treatment solutions at -20°C. Also keep the first and last steps of the dilution to validate the dosage.

2.4. Pesticide exposure

Starvation – day before pesticide exposure:

1. Place the box with adults in the fridge until the flies calm down.
2. Take individual (one by one) flies from the fridge, weigh individual flies by placing 2mL tube on a fine balance, record the weight of the fly at a precision of at least 0.1 mg alongside the specimen ID, date of emergence and sex in the respective excel template.
3. Place the individuals in a separate transparent plastic box (Fig 2B and C) with the appropriate ID at RT and natural light until exposure without food, and leave the individual to starve overnight.
4. According to input data in Excel doc and R script, individuals will be allocated for treatment (minimum 30 alive individuals per treatment).

On the day of exposure:

1. Expose the individuals to the treatment solution (20 µL) in the small plate during 3h.
2. After exposure change treatment solution with sucrose solution and leave individuals at RT exposed to natural light until the end of experiment.

2.5. Measurements and observation after treatment

1. Mortality checks at: 4h; 24h; 48h; 72h and 96h.
2. Weigh treatment solution consumption precisely.
3. Sublethal effects: unusual behavior; paralysis; reduced coordination.

For WP3, collect haemolymph and fat bodies for proteome studies and for transcriptome studies, preferably from the same specimens, using specimens living at 48 hrs post treatment. Details are to be provided in protocols from WP3. Collect a minimum of n=3 haemolymph samples per treatment and n = 3 fat body samples per treatment.The most sensitive species will initially be studied.

In case of *Eristalis arbustorum* at each stage of mortality check, collect dead individuals separately to Eppendorf tubes marked with a specific code (Species_Mass_T_MC_XX), freeze and send to PIWet for toxicokinetic analysis.

Codes should be entered as **Species_ Mass_T_MC_XX**

where:

Species – *Eristalis arbustorum* (EAr)
Mass - mass of the individual insect before pesticides exposure (in g)
T - Treatment Identifier (P, N, A, B, C, D, E, F, G or H)
MC – Mortality Check (4h, 24h, 48h, 72h or 96h)
XX – Amount of treated solution (µL)
Individuals after haemolymph collection also send to PIWet including additional mark on the label (H): Species_ Mass_T_M _XX**_H**
Individuals after fat bodies collection will not be tested by PIWet.

2.6. Raw data

Row data will be collected in an Excel file created within the group WP2. Data includes data before exposure which will be used for allocation individuals for treatment (such as stage, sex, body mass, and created ID for every individual), and after exposure (daily check of mortality and sublethal effects).

3. CHECKLIST

Schedule	Protocol	x
Day 1	**Reception and storage of pupae** ● Place at random 20 pupae per box.	
Days 2 (….)	**Daily check (8h)** ● Separate emerged adults in individual boxes. ● Transfer non-emerged pupae in a new box. ● Clean the box of frass.	
Days 3 (…..)	● The day before exposure, weigh the individual and leave them to starve overnight. **Exposure of individuals (10h)** ● Expose individuals to treatment solutions. ● Wait 3h. ● Weigh the treatment solutions.	

Days…..	**<u>Daily check (10h)</u>** ● Mortality (Remove dead or sick individuals). ● Renew the syrup solution. ● Clean the box of frass. ● Sublethal effects.	

WildPosh D2.4 Appendix 2: list of target spcies used in WP2

Bee species	Taxonomic family	MLU	UMONS	UFR	UNSPMF, BIOS	Developmental stages (males and females) for WP3	
Andrena vaga	Andrenidae					Larvae 3rd and 4th std	Adults
Anthophora plumipes	Apidae						
Colletes hederae	Colletidae						
Lassioglossum malachurum	Halictidae						
Osmia brevicornis	Megachilidae						
Osmia bicornis	Megachilidae						
Bombus hypnorum	Apidae					Larvae 3rd and 4th std	
Bombus lapidarius	Apidae						
Bombus pascuorum	Apidae						
Bombus terrestris	Apidae						

Lepidoptera species	Taxonomic family		UMONS			Developmental stages (males and females) for WP3	
Macroglossum stellatarum	Sphingidae					Caterpillars (Last stage)	Adults
Papilio machaon	Papilionidae						
Maniola jurtina	Nymphalidae						
Saturnia pavonia	Saturniidae						
Aglais urticae	Nymphalidae						

Hover fly species	Tribe				UNSPMF, BIOS	Developmental stages (males and females) for WP3	
Eristalis arbustorum	Eristalini					not larvae	Adults
Eristalinus aeneus	Eristalini						
Eupeodes corollae	Syrphini					potentially using larvae	
Myathropa florea	Eristalini					not larvae	
Cheilosia canicularis	Rhingiini						

Key: Responsible Partner

Date: 18.12.2024

WildPosh D2.4 Appendix 3A. Datasheets for recording data generated by the protocol for acute oral exposure.

Description	This file contains data on the WP2 **acute oral exposure** experiments to determine the sensitivity of pollinator species to several pesticides to the weight-adjusted LD50 of Bombus terrestris.				
Keywords	Keyword 1; Keyword 2; Keyword 3; Keyword 4				
Sheets	**Variables**	**ID**	**Pre-exposure**	**Post-exposure**	**[Enter sheet name]**
Sheet description	This is list of variables used in this datasets along with their definitions, and additional information.	This shows the individual elements of the ID variable	Here data collected before pesticide exposure are entered. These are used for the stratified random allocation of individuals to treatments	Here the data collected after pesticide allocation are entered.	[Enter description]
File manager	Enter the name of the person responsible for managing this particular file	[Enter the (hyperlinked) email address of the file manager]			
Project	WildPosh				
WPs	WP2				
Data owning partners	[Enter acronyms of data-owning/ involved partner organizations]				
Countries					
Date created					
Protocols	[Enter the name of a protocol used to generate these data; link protocol]	[Enter the name of a protocol used to generate these data; link protocol]			
Publications	[Enter the name of the citation of the publication]	[Enter the name of the citation of the publication]			
Publications DOI	[Enter the hyperliked DOI of the publication]	[Enter the DOI of the publication]			

Variable	Definition	Unit	Example		Drop_down	==>			
ID_unique	Code composed of Stage, Species, Sex and ID separated by underscore (automatically done by the excel file)		A_BT_F_1						
Stage	Adult (A) ; Larva (L).		A		A	L			
Species	Test species: Bombus terrestris (BT) ; Bombus hypnorum (BH) ; Bombus lapidarius (BL) ; Bombus pascuorum (BP) ; Andrena vaga (AV) ; Colletes hederae (CH) ; Anthophora plumipes (AP) ; Osmia brevicronis (OBR) ; Osmia bicornis (OBI) ; Halictus scabiosae (HS).		BT						
Sex	Female (F) ; Male (M).		F		F	M			
ID	Number of individual unique within the same stage of the same species of the same sex but not across different species/stages/sexes: 1, 2, 3, 4			4					
Origin	Origin of specimen. This could be either a site or the vender (in the case of Osmia)		Wildbiene und Partner						
Date_obtained	The date it was collected from the wild as adults or hatched from cocoons			23/04/2024					
Date_pre_assessment	The date the pre-assessment was done (i.e. determination of Body_mass, SC_pre_expo, Dead_pre_expo)			26/04/2024					
Age_pre_expo	Difference between Date_pre_assessment and Date_obtained in days	days		3					
Body_mass	Body mass of the individual at the start of the experiment (in g)	g		0.12					
Dead_pre_expo	Alive (0) ; Dead (1) in pre-assessment			0		1	0		
Sublethal_effs_pre_expo					Paralysis	Unusual behav	Reduced coordination	Other	None
Excluded_manually	Exluded (1) or not excluded (0 or blank) based on decision by file manager rather than the R script on treatment allocation			1					
Observer	Person taking the measurements		Alicia Kling						
Data_enterer	Person entering data in this excel file		Alicia Kling						
Comments	Comments, e.g. details on sublethal effects								
Treatment	P: positive control dimethoate. N: negative control solvent (acetone). A: Acetamiprid. B: Cypermethrin. C: Tebuconazole. D: Acetamiprid + Cypermethrin. E: Acetamiprid + Tebuconazole. F: Cypermethrin + Tebuconazole. G: Cypermethrin + Tebuconazole + Acetamiprid . H: field concentration (extreme value).		P						
Date_exposure	Date of exposure			01/05/2024					
T_syrup_offered	Volume of treated syrup offered in ml	mL		0.2					
TSC	Treated syrup consumption as a proportion of the whole dose (visually estimated). Value between 0 and 1			0.8					
Dead_after_4h	Alive (0) ; Dead (1) 4 hours after exposure			0		1	0		
Sublethal_effs_4h					Paralysis	Unusual behav	Reduced coordination	Other	None
Dead_after_24h	Alive (0) ; Dead (1) 24 hours after exposure			0		1	0		
Sublethal_effs_24h					Paralysis	Unusual behav	Reduced coordination	Other	None
Dead_after_48h	Alive (0) ; Dead (1) 48 hours after exposure			0		1	0		
Sublethal_effs_48h					Paralysis	Unusual behav	Reduced coordination	Other	None
Dead_after_96h	Alive (0) ; Dead (1) 96 hours after exposure			1		1	0		
Sublethal_effs_96h					Paralysis	Unusual behav	Reduced coordination	Other	None
SC_after_96h	Total syrup consumption after exposure								
Box_ID	ID of box in which the individuals were group housed								
Date_acclimatization_start	Date at which the acclimatization period started								
Time_acclimatization_start	Time of day at which the acclimatization period started								
Batch	ID idetifying the subset of individuals that are receiving exposure on the same day								
N_individuals_indicated_sex	The number of individuals of the indicated sex that are group housed together			16					
N_individuals_other_sex	The number of individuals that are removed from the box because of wrong sex			2					
N_dead_8h	The number of individuals that are dead after 8 hours			3					
N_dead_2d	The cumulative number of individuals that are dead after 2 days			1					
N_alive_8h	The number of individuals that remain alive after 8 hours (within the regarded box) [automatically calculated]			15					
N_alive_2d	The number of individuals that remain alive after 2 days (wihin the regarded box) [automatically calculated]			13					
SC_8h_mL	Syrup consumption during 8 hours								
SC_2d_mL	Syrup consumption during 2 days								
mean_SC_per_bee_8h_mL	Mean syrup consumption per bee in acclimatization period over 8 hours [automatically calculated]	mL							
mean_SC_per_bee_2d_mL	Mean syrup consumption per bee in acclimatization period over 2 days [automatically calculated]	mL							
Group_weight_pre_starvation	Collective weight of individuals that are starved together								
mean_body_mass_pre_starvation	Mean body mass per individual before starvation [automatically calculated]	g							
overall_mean_body_mass_pre_starvation	Mean body mass per individual before starvation across all individuals of the same species and sex [automatically calculated]	g							
overall_mean_body_mass_post_starvation	Mean body mass per individual after starvation across all individuals of the same species and sex [automatically calculated]	g							
overall_mean_SC_8h_mL	Mean syrup consumption per individual in acclimatization period over 2 days [automatically calculated]	mL							
overall_mean_SC_2d_mL	Mean syrup consumption per indivdual in acclimatization period over 8 hours [automatically calculated]	mL							
overall_mean_SC_per_d_mL	Mean syrup consumption per individual and per day in acclimatization period (averaged over two days) [automatically calculated]	mL							
overall_mean_SC_2d_mL_corrected	Mean syrup consumption per individual in acclimatization period over 2 days (corrected for number of individuals that were alive on day one) [automatically calculated]	mL							

Box_ID	Species	Sex	Date_acclimatization_start	Time_acclimatization_start	Date_exposure	Date_obtained	Batch	N_individuals_indicated_sex	N_individuals_other_sex	N_dead_8h	N_dead_2d	N_dead_3d	N_alive_8h	N_alive_2d	N_alive_3d	SC_8h_mL	SC_2d_mL	SC_2nd_day_mL	mean_SC_per_bee_8h_mL	mean_SC_per_bee_2d_mL	mean_SC_per_bee_2nd_day_mL	mean_SC_per_bee_per_d_mL	Group_weight_pre_starvation	mean_body_mass_pre_starvation	Used_in_experiment	Comments

ID_unique	Stage	Species	Sex	ID	Origin	Date_obtained	Date_pre_assessment	Age_pre_expo	Body_mass	Dead_pre_expo	Sublethal_effs_pre_expo	Box_ID	Excluded_manually	Observer	Data_enterer	Comments

Treatment	ID_unique	Stage	Species	Sex	ID	Date_exposure	Time_exposure	T_syrup_offered	TSC	Dead_after_0h	Sublethal_effs_0h	Dead_after_4h	Sublethal_effs_4h	SC_after_4h	Dead_after_24h	Sublethal_effs_24h	SC_after_24h	Dead_after_48h	Sublethal_effs_48h	SC_after_48h	Dead_after_72h	Sublethal_effs_72h	SC_after_72h	Dead_after_96h	Sublethal_effs_96h	SC_after_96h	Observer	Data_enterer	Comments

Species	Sex	overall_mean_body_mass_pre_starvation	overall_mean_body_mass_post_starvation	overall_mean_SC_8h_mL	overall_mean_SC_2d_mL	overall_mean_SC_per_d_mL	overall_mean_SC_2d_mL_corrected

WildPosh D2.4 Appendix 3B. Datasheets for recording data generated by the protocol for acute topical exposure.

Description	This file contains data on the WP2 **acute oral exposure** experiments to determine the sensitivity of pollinator species to several pesticides to the weight-adjusted LD50 of Bombus terrestris.				
Keywords	Keyword 1; Keyword 2; Keyword 3; Keyword 4				
Sheets	**Variables**	**ID**	**Pre-exposure**	**Post-exposure**	**[Enter sheet name]**
Sheet description	This is list of variables used in this datasets along with their definitions, and additional information.	This shows the individual elements of the ID variable	Here data collected before pesticide exposure are entered. These are used for the stratified random allocation of individuals to treatments	Here the data collected after pesticide allocation are entered.	[Enter description]
File manager	Enter the name of the person responsible for managing this particular file	[Enter the (hyperlinked) email address of the file manager]			
Project	WildPosh				
WPs	WP2				
Data owning partners	[Enter acronyms of data-owning/ involved partner organizations]				
Countries					
Date created					
Protocols	[Enter the name of a protocol used to generate these data; link protocol]	[Enter the name of a protocol used to generate these data; link protocol]			
Publications	[Enter the name of the citation of the publication]	[Enter the name of the citation of the publication]			
Publications DOI	[Enter the hyperliked DOI of the publication]	[Enter the DOI of the publication]			

Variable	Definition	Unit	Example		Drop_down	==>			
ID_unique	Code composed of Stage, Species, Sex and ID separated by underscore (automatically done by the excel file)		A_BT_F_1						
Stage	Adult (A) ; Larva (L).		A		A	L			
Species	Test species: Bombus terrestris (BT) ; Bombus hypnorum (BH) ; Bombus lapidarius (BL) ; Bombus pascuorum (BP) ; Andrena vaga (AV) ; Colletes hederae (CH) ; Anthophora plumipes (AP) ; Osmia brevicronis (OBR) ; Osmia bicornis (OBI) ; Halictus scabiosae (HS).		BT						
Sex	Female (F) ; Male (M).		F		F	M			
ID	Number of individual unique within the same stage of the same species of the same sex but not across different species/stages/sexes: 1, 2, 3, 4			4					
Origin	Origin of specimen. This could be either a site or the vender (in the case of Osmia)		Wildbiene und Partner						
Date_obtained	The date it was collected from the wild as adults or hatched from cocoons			23/04/2024					
Date_pre_assessment	The date the pre-assessment was done (i.e. determination of Body_mass, SC_pre_expo, Dead_pre_expo)			26/04/2024					
Age_pre_expo	Difference between Date_pre_assessment and Date_obtained in days	days		3					
Body_mass	Body mass of the individual at the start of the experiment (in g)	g		0.12					
Syrup_pre_expo_offered	Volume of syrup offered before exposure in ml	ml		0.2					
SC_pre_expo	Syrup consumption before exposure as a proportion of the whole dose (visually estimated). Value between 0 and 1.			0.9					
Dead_pre_expo	Alive (0) ; Dead (1) in pre-assessment			0		1	0		
Sublethal_effs_pre_expo					Paralysis	Unusual behav	Reduced coordination	Other	None
Excluded_manually	Exluded (1) or not excluded (0 or blank) based on decision by file manager rather than the R script on treatment allocation			1					
Observer	Person taking the measurements		Alicia Kling						
Data_enterer	Person entering data in this excel file		Alicia Kling						
Comments	Comments, e.g. details on sublethal effects								
Treatment	P: positive control dimethoate. N: negative control solvent (acetone). A: Acetamiprid. B: Cypermethrin. C: Tebuconazole. D: Acetamiprid + Cypermethrin. E: Acetamiprid + Tebuconazole. F: Cypermethrin + Tebuconazole. G: Cypermethrin + Tebuconazole + Acetamiprid . H: field concentration (extreme value).		P						
Date_exposure	Date of exposure			01/05/2024					
Dead_after_4h	Alive (0) ; Dead (1) 4 hours after exposure			0		1	0		
Sublethal_effs_4h					Paralysis	Unusual behav	Reduced coordination	Other	None
Dead_after_24h	Alive (0) ; Dead (1) 24 hours after exposure			0		1	0		
Sublethal_effs_24h					Paralysis	Unusual behav	Reduced coordination	Other	None
Dead_after_48h	Alive (0) ; Dead (1) 48 hours after exposure			0		1	0		
Sublethal_effs_48h					Paralysis	Unusual behav	Reduced coordination	Other	None
Dead_after_96h	Alive (0) ; Dead (1) 96 hours after exposure			1		1	0		
Sublethal_effs_96h					Paralysis	Unusual behav	Reduced coordination	Other	None
SC_after_96h	Total syrup consumption after exposure								
Box_ID	ID of box in which the individuals were group housed								
Date_acclimatization_start	Date at which the acclimatization period started								
Time_acclimatization_start	Time of day at which the acclimatization period started								
Batch	ID idetifying the subset of individuals that are receiving exposure on the same day								
N_individuals_indicated_sex	The number of individuals of the indicated sex that are group housed together			16					
N_individuals_other_sex	The number of individuals that are removed from the box because of wrong sex			2					
N_dead_8h	The number of individuals that are dead after 8 hours			3					
N_dead_2d	The cumulative number of individuals that are dead after 2 days			1					
N_alive_8h	The number of individuals that remain alive after 8 hours (within the regarded box) [automatically calculated]			15					
N_alive_2d	The number of individuals that remain alive after 2 days (wihin the regarded box) [automatically calculated]			13					
SC_8h_mL	Syrup consumption during 8 hours								
SC_2d_mL	Syrup consumption during 2 days								
mean_SC_per_bee_8h_mL	Mean syrup consumption per bee in acclimatization period over 8 hours [automatically calculated]	mL							
mean_SC_per_bee_2d_mL	Mean syrup consumption per bee in acclimatization period over 2 days [automatically calculated]	mL							
overall_mean_body_mass_post_starvation	Mean body mass per individual after starvation across all individuals of the same species and sex [automatically calculated]	g							
overall_mean_SC_8h_mL	Mean syrup consumption per individual in acclimatization period over 2 days [automatically calculated]	mL							
overall_mean_SC_2d_mL	Mean syrup consumption per indivdual in acclimatization period over 8 hours [automatically calculated]	mL							
overall_mean_SC_per_d_mL	Mean syrup consumption per individual and per day in acclimatization period (averaged over two days) [automatically calculated]	mL							
overall_mean_SC_2d_mL_corrected	Mean syrup consumption per individual in acclimatization period over 2 days (corrected for number of individuals that were alive on day one) [automatically calculated]	mL							

Box_ID	Species	Sex	Date_acclimatization_start	Time_acclimatization_start	Date_exposure	Date_obtained	Batch	N_individuals_indicated_sex	N_individuals_other_sex	N_dead_8h	N_dead_2d	N_alive_8h	N_alive_2d	SC_8h_mL	SC_2d_mL	SC_2nd_day_mL	mean_SC_per_bee_8h_mL	mean_SC_per_bee_2d_mL	mean_SC_per_bee_2nd_day_mL	mean_SC_per_bee_per_d_mL	Comments

ID_unique	Stage	Species	Sex	ID	Origin	Date_obtained	Date_pre_assessment	Age_pre_expo	Body_mass	Dead_pre_expo	Sublethal_effs_pre_expo	Box_ID	Excluded_manually	Observer	Data_enterer	Comments

Treatment	ID_unique	Stage	Species	Sex	ID	Date_exposure	Time_exposure	Dead_after_4h	Sublethal_effs_4h	SC_after_4h	Dead_after_24h	Sublethal_effs_24h	SC_after_24h	Dead_after_48h	Sublethal_effs_48h	SC_after_48h	Dead_after_72h	Sublethal_effs_72h	SC_after_72h	Dead_after_96h	Sublethal_effs_96h	SC_after_96h	Observer	Data_enterer	Comments

Species	Sex	overall_mean_body_mass_post_starvation	overall_mean_SC_8h_mL	overall_mean_SC_2d_mL	overall_mean_SC_per_d_mL	overall_mean_SC_2d_mL_corrected

WildPosh D2.4 Appendix 4. R-scripts for initial processing of data generated by the protocols.

```r
################################################################################
!
# Script to form groups with low between-group variance,
# which are randomly assigned to different treatments
################################################################################
!

library(anticlust); library(readxl)
library(plyr); library(tidyverse)
library(openxlsx)

# Read data and exclude unsuitable individuals
path_data_in <- file.choose()
path_data_out <- gsub(".xlsx", "_treatments.xlsx", path_data_in)

pre_data = read_excel(path_data_in, sheet = "Pre_exposure") %>%
  filter(Alive_pre_expo == 1 & (
    is.na(Excluded_manually) == T | Excluded_manually != 1
  ))

# Define covariates and treatments
covariates = c("Body_mass", "Age_pre_expo", "SC_pre_expo")

treatments = c("N", "P", "A", "B", "C", "D", "E", "F", "G", "H")
# treatments = c("NC", "PC", "T1", "T2", "T3", "T4", "T5", "T6", "T7",
"T8")

treatments_shuffled = sample(treatments)

n_treatments = length(treatments)
n_replicates = floor(nrow(pre_data) / n_treatments)

summary(pre_data)

# Determine experimental individuals and spare individuals (those are
sorted out)
pre_data$set_similar_individuals <- matching(
  x = scale(pre_data[, covariates]),
  p = n_treatments,
  match_within = pre_data$Sex
  )

experimental_individuals = subset(pre_data, set_similar_individuals <=
n_replicates)

```

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# Spare colonies are sorted out
spare_individuals = subset(pre_data, set_similar_individuals > n_replicates | is.na(set_similar_individuals) == T)
spare_individuals$set_similar_individuals = NULL
spare_individuals$Treatment = "Spare individual"
spare_individuals <- spare_individuals %>%
  dplyr::select(Treatment, everything())

# Individuals of the same set are then distributed to all the different treatment groups.
experimental_individuals$Treatment <- anticlustering(
  experimental_individuals[, covariates],
  K = n_treatments,
  categories = experimental_individuals$set_similar_individuals,
  objective = "variance",
  method = "exchange",
  repetitions = 1000
)

experimental_individuals$Treatment = mapvalues(experimental_individuals$Treatment,
                                               from = 1:n_treatments,
                                               to = treatments_shuffled)

experimental_individuals$set_similar_individuals = NULL

experimental_individuals <- experimental_individuals %>%
  dplyr::select(Treatment, everything())

# Create a new Excel workbook
wb <- createWorkbook()

# Add sheets to the workbook
addWorksheet(wb, "Experimental_individuals")
addWorksheet(wb, "Spare_individuals")

# Write data to the sheets
writeData(wb, "Experimental_individuals", experimental_individuals)
writeData(wb, "Spare_individuals", spare_individuals)

# Save the workbook to a file
saveWorkbook(wb, path_data_out, overwrite = TRUE)


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82
83
84
85
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