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## Diverse pollen nutrition can improve the development of solitary bees but does not mitigate negative pesticide impacts

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#### Abstract

Floral resource loss and pesticide expecter are major threats to bees in intensively managed agroecosystems, but interactions and the drivers remain poorly understood. Altered composition and lowered diversity of polle. Invition may reinforce negative pesticide impacts on bees. Here we investigated the development and survival of the solitary bee *Osmia bicornis* provisioned with three different pollen types, as well as a mixture of these types representing a higher pollen diversity. We exposed bees of each nutritional treatment to five pesticides at different concentrations in the laboratory. Two field-realistic concentrations of three nicotinic acetylcholine receptor (nAChR) modulating insecticides (thiacloprid, sulfoxaflor and flupyradifurone), as well as of two fungicides (azoxystrobin and tebuconazole) were examined. We further measured the expression of two detoxification genes (*CYP9BU1, CYP9BU2*) under exposure to thiacloprid across different nutrition treatments as a potential mechanistic pathway driving pesticide-nutrition interactions. We found that more diverse pollen nutrition reduced development time, enhanced pollen efficacy (cocoon weight divided by consumed pollen weight) and pollen consumption, and increased weight of *O. bicornis* after larval development

(cocoon weight). Contrary to fungicides, high field-realistic concentrations of all three insecticides negatively affected *O. bicornis* by extending development times. Moreover, sulfoxaflor and flupyradifurone also reduced pollen efficacy and cocoon weight, and sulfoxaflor reduced pollen consumption and increased mortality. The expression of detoxification genes differed across pollen nutrition types, but was not enhanced after exposure to thiacloprid. Our findings highlight that lowered diversity of pollen nutrition and high field-realistic exposure to nAChR modulating insecticides negatively affected the development of *O. bicornis*, but we found no mitigation of negative pesticide impacts through increased pollen diversity. These results have important implications for risk assessment for bee pollinators, indicating that negative effects of nAChR modulating insecticides to developing solitary bees are currently underestimated.

## Keywords

*Osmia bicornis*, pollen nutrition diversity, per iciae exposure, interactive effects, solitary bee development, detoxification gene expression

## **1. Introduction**

Bees, the most important pollinaters or entomophilous flowering wild plants and crops, are dependent on suitable and abundant theral resources for sustaining their development and reproduction (Brodschneider and Cralinheim 2010, Leach and Drummond 2018). In recent years, alarming declines of wild bees have been reported (Potts et al. 2010, Zattara and Aizen 2021), which have been linked to the loss of appropriate and diverse floral resources as consequence of habitat loss and agricultural intensification (e.g., Scheper et al. 2014, Goulson et al. 2015, Parreño et al. 2021). Additionally, the high use of pesticides in intensive agriculture is considered an important driver of bee decline (IPBES 2016, Dicks et al. 2021). Together, these stressors may cause additive or synergistic negative effects on bees (Dance et al. 2017, Tosi et al. 2017, Siviter et al. 2021), thereby jeopardizing biodiversity, ecosystem functioning and crop pollination services (IPBES 2016). Research on western honeybees (*Apis mellifera*) were developed to a large extent on this topic but such potential interactions of unsuitable

nutrition and pesticide exposure on different live stages of wild bees and their underlying mechanisms are not well understood (Wood et al. 2020).

Bees are fully dependent on nectar and pollen from flowering plants and a suitable nutrition is key, e.g., for survival and immunity (Alaux et al. 2010). Nectar is the main source of energy in form of carbohydrates, whereas pollen offers essential micro- and macronutrients, e.g., proteins, lipids, vitamins, starch, sterols and minerals (Vaudo et al. 2015). For instance, proteins and amino acids play an important role in development, body size and adult reproduction (Tasei and Aupinel 2008, Vanderplanck et al. 2014, Archer et al. 2021). Bees can balance their nutritional int ke Behmer 2009, Leonhardt and Blüthgen 2012) by collecting pollen from different plant taxa and p ovisioning a mixture of pollen to their offspring (Eckhardt et al. 2014, Bukovinszky et al. 2017, Filipiak 2019) to obtain an optimal diet and maintain relatively constant protein to lipid ratios (V.udo et al. 2016, Vaudo et al. 2020, Peters et al. 2022). The mixing of pollen can also be advant genus by increasing foraging efficiency (e.g., when females also collect pollen from flowers th' y m ainly visit for nectar) or by allowing less favourable pollen types to be used by mixing then, with higher quality pollen (Williams and Tepedino 2003, Eckhardt et al. 2014). Several studies have investigated the effects of mono-floral (low diversity) and poly-floral (higher diversity) poller notificition on bee development, mainly on honeybees and bumblebees (Parreño et al. 2021). While some . udies suggest that a mixed pollen nutrition is beneficial (e.g., Tasei and Aupinel 2008, Dance  $\epsilon$  al. 017), others suggest that it is not pollen diversity *per se*, but rather the balanced availability of esse tial nutrients is the key factor for bees (Bukovinszky et al. 2017, Moerman et al. 2017, Carnell et al. 2020, Filipiak et al. 2022).

Nutrition plays an important role in shaping bees' capacity to mitigate negative effects of pesticides (Wahl and Ulm 1983, Di Pasquale et al. 2013, Schmehl et al. 2014, Crone and Grozinger 2021). The high use of pesticides associated with intensive agriculture can lead to the contamination of pollen and nectar of treated crops, but residues are also found in wild plants near crop fields (Zioga et al. 2020). The detoxification of pesticides is energetically costly, making high quality nutrition key for this process (Berenbaum and Johnson 2015). In honeybees, intake of pollen has been demonstrated to reduce the

toxicity of pesticides (Wahl and Ulm 1983, Barascou et al. 2021). In fact, diets of suitable quality and quantity of proteins and lipids can enhance the expression of detoxification genes and may increase the survival of insecticide-exposed honeybees (Schmehl et al. 2014, Crone and Grozinger 2021). The ingestion of secondary metabolites can further upregulate the expression of detoxification genes and thereby modulate the sensitivity to pesticides (Johnson et al. 2012, Ardalani et al. 2021). Pesticides, in turn, can adversely affect food consumption rates, food preferences and foraging success of bees, which potentially further augments the negative impacts of nutritional stress (Kessler et al. 2015, Stanley and Raine 2016, Sgolastra et al. 2018, Vodovnik et al. 2021).

Larvae of wild solitary bees can be exposed to pesticides mainly via the consumption of or contact with contaminated pollen provisions (Eeraerts et al. 2020, Sgolast., et al. 2020) or nesting material (i.e., mud, Fortuin et al. 2021). Risk assessment of pesticides on larva' stap s of bees is currently restricted to social honeybees and bumblebees, while risk assessment a d candard protocols for solitary bee larvae are lacking (Sgolastra et al. 2020), although recommendations for such tests have recently been proposed (Eeraerts et al. 2020). Yet, solitary bees L'ffer in life history and physiological traits from social bees, which may result in different routes an 1'evels of exposure, as well as different sensitivity associated with distinct levels and pathways on metabolization of compounds and their detoxification (Arena and Sgolastra 2014, Beadle et al. 2019, Sgolastra et al. 2019). For example, social bees typically continuously feed their larvae at d provide brood care, while solitary bee females provide their offspring once with a single pollen-lectar provision without any further brood care. Several studies have investigated the effects of different pollen nutrition (Praz et al. 2008, Sedivy et al. 2011, Filipiak and Filipiak 2020, Lawson et al. 2021, Filipiak et al. 2022) or pesticide exposure (Huntzinger et al. 2008, Hodgson et al. 2011, Sgolastra et al. 2015, Nicholls et al. 2017, Anderson and Harmon-Threatt 2019, Claus et al. 2021, Mokkapati et al. 2021) on solitary bee larval development. Of the studies that have investigated effects of pesticides on solitary bee development, most focused on impacts of neonicotinoid insecticides, with mixed results: some of them reported negative impacts on survival, development time and body weight (Anderson and Harmon-Threatt 2021, Mokkapati et al. 2021), while others found no adverse effects (Nicholls et al. 2017). Studies on fungicides are rare and their potential impacts on

solitary bee development remain poorly understood (Huntzinger et al. 2008, Kopit et al. 2022). Importantly, to our knowledge, potential interactive effects between different pollen nutrition types and pesticide exposure, as well as the role of pollen diversity in the mitigation of negative pesticide impacts on solitary bee development remain largely unexplored (Kopit et al. 2022).

In the present experimental study, we used experiments in factorial designs to examine the main and interactive effects of different pollen nutrition types and field-realistic exposure to pesticides on the development and mortality of the solitary bee Osmia bicornis. For this purpose, O. bicornis larvae were reared on four pollen diet treatments, three treatments with low-dir ersi y pollen types (i.e., dominated by one or two pollen species) and one treatment with higher-di ersity pollen (i.e., mixture of the three first pollen diets). The pollen provisions were treated with one of five pesticides: three nicotinic acetylcholine receptor (nAChR) modulating insecticides (the 'loprid, sulfoxaflor or flupyradifurone) and two commonly used fungicides (azoxystrobin on eb conazole). We assessed 1) how the different nutrition types affect O. bicornis development ti ne, pollen consumption, pollen efficacy (i.e., the rate at which consumed pollen is transformed into body weight), body weight after larval development (cocoon weight) and survival until adul c mergence and specifically, whether the high diversity pollen treatment performed better comprest to the low diversity pollen treatments; 2) how the exposure to pesticides affects these endpoints, 3) how pesticide effects are modulated by different pollen types, specifically whether potent al negative impacts of high pesticide exposure can be mitigated by increased diversity in the pollen diet, and finally 4) how two detoxification genes (CYP9BU1, CYP9BU2) are expressed in larvae exposed to the insecticide thiacloprid across different nutrition treatments.

#### 2. Methods

#### 2.1. Study design

We assessed the impacts of pesticides and nutrition on the development of the solitary bee *O. bicornis* in a laboratory study from April 2020 to April 2021 at Agroscope in Zürich, Switzerland. The Red mason bee, *O. bicornis*, is a cavity-nesting, polylectic, univoltine bee species active in spring and mainly distributed in Europe (Westrich 2019). For this purpose, *O. bicornis* eggs freshly laid by freely foraging

females in spring 2020 were transferred and reared under controlled conditions in the laboratory on pollen provisions (see below) receiving different experimental treatments (different combinations of pollen nutrition and pesticide exposures). Five different pesticides were used in the experiment: three insecticides, i.e., the neonicotinoid thiacloprid, the sulfoximine sulfoxaflor and the butenolide flupyradifurone, as well as the two commonly used fungicides azoxystrobin (β-methoxyacrylate) and tebuconazole (triazole). Two concentrations were tested for each pesticide and, as they were solved in acetone, a water-acetone-control treatment was used in addition to a water-only-control. For each of the five pesticides, a factorial design was implemented, including all combinations of the four pesticide treatment levels (low concentration, high concentration, water-only convol, water-acetone-control) and the four nutrition types (three different pollen types and mixture of the est types). A sample size of 30 female eggs per treatment combination was used and the used and the in total.

#### 2.2. Preparation of pollen provisions s' ike I with pesticides

**Nutrition treatments.** Certified organic honeybee-collected pollen was purchased from Abeille heureuse<sup>®</sup>, France, and subsequently gain na irradiated in order to prevent contamination by pathogens and parasites. Four different nutrition treatments were used, including three "low diversity" types dominated by a single or two polle. species: (1) *Cistus ladanifer* (95%; "C"), (2) *Salix* spp. (89%; "S") and (3) *Brassicaceae/Quervus* (71% (36% + 35%, respectively); "BQ") and (4) a mixture ("MIX") of these three low diversity pollen (ratio of 1:1:1 by weight), yielding a higher-diversity pollen diet. The composition of pollen types was palynologically analysed and confirmed (Barraud et al. 2022). The three different low-diversity pollen types were chosen based on differences in nutritional properties such as protein, lipid and amino acid contents, which was hypothesized to result in positive nutritional complementary effects in the 'MIX' treatment (see Supplementary Table 1 and Barraud et al. 2022 for a detailed description of nutritional compositions of pollen types).

**Pesticide concentrations tested**. The tested concentrations of pesticides in pollen were determined based on published data of residue levels in bee-collected pollen. The high concentration was chosen to

reflect a worst-case field-realistic scenario (i.e., exposure shortly after spray application of a pesticide) according to highest residue values reported for bee-collected pollen in the literature (see below). The low concentration corresponded to a 10-fold lower value, which was generally within the higher range of most commonly reported residue values. The following high concentrations were used: sulfoxaflor: 3 mg/kg (EFSA 2019); thiacloprid: 0.75 mg/kg (Pohorecka et al. 2013, Böhme et al. 2018); azoxystrobin 1.9 mg/kg (Observatory of Pesticide Residue, ITSAP - Institut de l'Abeille 2014 (personal communication), Rennich et al. 2013); tebuconazole: 0.5 mg/kg (Böhme et al. 2018). For flupyradifurone, 40 mg/kg was used as high concentration based on esidue unit doses (RUD), i.e., the residue level (ca. 340 mg/kg after foliar application; EFSA 2017) livided by the recommended application rate for Sivanto<sup>®</sup> Prime in apple (Bayer Crop Science Austria 2022). The pesticides azoxystrobin (PESTANAL<sup>TM</sup> analytical standard, 100 mg, Sigma Aldrich<sup>®</sup>), flupyradifurone (PESTANAL<sup>TM</sup> analytical standard, 100 mg, Sigma Aldrich<sup>®</sup>), <sup>t</sup>ebuconazole (PESTANAL<sup>TM</sup> analytical standard, 250 mg, Sigma Aldrich<sup>®</sup>) and thiacloprid (1<sup>™</sup> S<sup>7</sup> ANAL<sup>™</sup> analytical standard, 100 mg, Sigma Aldrich<sup>®</sup>) were purchased from Dr. Grogg C<sup>1</sup> emj. AG, Switzerland. Sulfoxaflor (10 mg) was purchased from Greyhound Chromatography And Allied Chemicals Ltd, UK. The content of acetone was adjusted so that it was identical in all pesticide solutions (0.46%). A separate water-acetone-control solution (without any pesticide) with this a otone content was additionally prepared. A water-only-control (without acetone) was also prepare<sup>1</sup> Stock solutions, pesticide and control solutions were stored at -20 °C. For each pesticide-nut itio1 treatment combination, 20 g of pollen provision mass (spiked with pesticides, water only, or witer and acetone) was prepared. For this purpose, 18 g honeybee-collected pollen pellets were finely ground in a mortar, and 2 g (1.74 mL) of pesticide-sugar solution (1 mL pesticide or control solution + 0.74 mL 77% (w/v) sugar solution) were added in 4 steps of 0.435 mL each. For the sugar solution, commercially available household sugar (sucrose) was used. After each step, the pollen was mixed in the mortar for 1 minute by hand to obtain a homogeneous distribution of the pesticide. Then, 30 separate pollen provisions, each weighing 400 mg (based approximately on Bosch and Vicens 2002), were formed by hand. These provisions were stored at -20 °C until they were used for the experiment.

#### 2.3. Egg collection and assessments of bee development

At the beginning of April until mid-May 2020 a total of four batches of each roughly 2,000 cocoons of *O. bicornis* (males and females mixed; purchased from Wildbiene + Partner AG, Switzerland) were transferred, at intervals of ca. ten days, from a cold room (3 °C) to a sheltered place outdoors near the laboratory containing forty custom-made wooden nesting aids. Each nesting aid consisted of ten stacked, removable wooden layers each containing 13 semi-circular drilled cavities (130 cavities per nesting aid in total), in which *O. bicornis* females could nest (Supplementary Fig. 1). Two weeks after the release of the first batch, the first freshly laid O. *bicornis* eggs could be collected from the nests for the experiment.

The collection of O. bicornis eggs for the experiment was performed on a total of ten days between 17 April to 5 May 2020. Every morning before the collection on freshly laid eggs from the nesting aids, custom-made wooden plates with artificial brood coils and containing a set of different pollen provision treatments (prepared as described above) we - pr spared in the laboratory. On each plate, nine randomly chosen provisions were carefully placed into the designated indentations. On top of each pollen provision, a small hollow was formed with a spatula for the egg to be placed in. Only eggs considered as female eggs based on their position in the nest, as well as on the size of the pollen provisions (Ivanov 2006, Seidelmann et al. 2010) we. collected (Supplementary Fig. 2). For harvesting, the eggs were carefully transferred onto tl e prepared pollen provisions on the custom-made wooden plates using a wet brush. After the transfer, they were covered with a transparent acetate sheet to prevent desiccation (Supplementary Fig. 3) and kept in an incubator under controlled conditions at 23 °C and 60% relative humidity during their development. This setup was intended to mimic the exposure of developing O. bicornis to pesticides in pollen under natural conditions. Pollen spiked with field-realistic pesticide concentrations was provided to each individual once at the egg stage (i.e., the egg was positioned on top of the pollen), as done by provisioning female *O. bicornis* in nature. In the following period of approx. 3-4 weeks the larvae hatched, continuously fed on the provided pollen provisions and subsequently started to spin their cocoon for metamorphosis. During this time period, larvae were exposed to the pesticides in pollen via feeding and physical contact. The developing bees were checked daily (except

on Sundays) for progress in their development during the entire experiment. The following stages were assessed by visual inspection: egg, larva not yet feeding, feeding larva, feeding and defecating larva, spinning larva, cocoon. If a larva was found dead, the date of death was noted. Larvae that did not hatch from the egg at the start of the experiment were not considered for further analysis, as it was likely that these eggs were damaged during the egg collection and transfer process. After completion of the cocoon spinning phase, the bees were still kept inside the incubator without further disturbance until mid-September 2020, in order to let them complete their metamorphosis inside the cocoons (Bosch et al. 2008). The amount (weight) of not consumed pollen (pollen leftor er) of the originally provisioned pollen was assessed for each bee. In some pollen provision leftove s pullen mites were detected (after bees had formed cocoons). As this could have potentially affected pullen leftover weight, we excluded infected leftovers from this analysis, and only analysed leftor weight no mites or negligible infestation levels.

#### 2.4. Overwintering and assessment of Fine gence rates

After the completion of metamorphosis, each cocoon was carefully cleaned and weighed, put into an individually labelled 2 mL Eppendorf t by vich a small hole and put back into the incubator. Then, the temperature in the incubator was copped to 15 °C on 22 September and further to 10 °C on 25 September to mimic natural pre-wintering conditions. For subsequent overwintering, the cocoons were carefully packed into card oard boxes at beginning of October 2020 and placed at a sheltered space outdoors on the experimental field station of Agroscope in Zürich. In the following spring (April 2021), cocoons were transferred to the laboratory where adult bees emerged at room temperature inside their tubes. The emergence of the bees was checked daily, and the sex of both emerged and not emerged bees was determined. In total, 1,741 bees were identified as females (83%), 208 as males (9.9%), while the sex of 148 bees could not be determined, because they died in an early development stage (7.1%). Depending on the analysed response variable, only female bees or bees of both sexes were included in the analyses (see statistical analysis section). Shortly after a bee emerged, it was released outdoors.

#### 2.5. Expression of detoxification genes

We measured the gene expression of two detoxification genes, *CYP9BU1* and *CYP9BU2*, in larvae exposed or not exposed to the neonicotinoid insecticide thiacloprid reared on different types of pollen provisions. These genes are known to be involved in the detoxification of thiacloprid in *O. bicornis* (Beadle et al. 2019). For this purpose, an additional 20 larvae per treatment were reared as described above on pollen provisions of each of the following six treatment combinations: *Cistus* (C) pollen, *Brassicaceae/Quercus* (BQ) pollen or MIX (*Cistus + Salix* (S) + *Brassicaceae/Quercus*) pollen, spiked with the high thiacloprid concentration (0.75 mg/kg) or with water-acetone-control (no pesticide). Due to the limited availability of resources, we chose to include only the 1 w diversity pollen types with the lowest and highest protein and amino acid contents (C and BQ), : s well as the MIX type, but not S. Larvae were sampled for gene expression analysis 12 days after they hatched from the egg by transferring them into labelled 1.5 mL Eppendorf tubes and firezing them at -80 °C.

Gene expression analysis was conducted at INRAF A ignon. Individual larvae were homogenized in 1 ml of Trizol reagent (Invitrogen®) with a T<sup>\*</sup>-sue\_yser (Qiagen®) (4x30 s at 30 Hz). The mixture was incubated for 5 min at room temperature and after centrifugation (12,000 g for 30 s. at 4 °C) the supernatant was used for RNA extractic 1. which was carried out as indicated in the Purelink RNA mini kit with on-column DNase I treatme. (Thermo Fisher Scientific®). For cDNA synthesis, 1,000 ng of RNA per sample were reverse-unscribed using the High capacity RNA to cDNA Kit (Applied Biosystems<sup>®</sup>). cDNA samples vere diluted ten-fold in molecular grade water. The expression level of CYP9BU1 and CYP9BU2 w s quantified and normalized to the housekeeping genes Elongation Factor  $\alpha 1$  (*EF* $\alpha 1$ ), Elongation Factor  $\gamma 1$  (*EF* $\gamma 1$ ) and Ribosomal Protein S5 (*RPS5*). For *EF* $\alpha 1$  and *EF* $\gamma 1$  we used sequences of primers previously published (Beadle et al. 2019). The sequence information of RPS5 was obtained from the NCBI database. Primers were designed using Primers3 (https://primer3.ut.ee/) and Primers Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and verified with PREMIER Biosoft (http://www.premierbiosoft.com/NetPrimer/AnalyzePrimer.jsp). We used the following primers for RPS5 (XM\_029192865): forward: TATTATGTACTGGTGCTAGG, reverse: TTGTATGTTGTCAAAAGAAC (amplicon size: 182 pb). Expression analysis was performed by quantitative PCR using a CFX 384 Biorad and the SYBR green detection method: 5 minutes at 95°C

followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, and a melting curve analysis with a temperature ramp from 55°C to 95°C. Three  $\mu$ l cDNA were mixed to 5 $\mu$ l SsoAdvanced<sup>TM</sup> Universal SYBR® Green Supermix (Biorad®), 1  $\mu$ l of forward primer (10  $\mu$ mol) and 1  $\mu$ l of reverse primer (10  $\mu$ mol). To verify the amplification specificity of the primer pairs, the melting curve was checked and PCR products were sequenced by Genoscreen (France) and analysed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/) and NCBI Blast (https://www.ncbi.nlm.nih.gov/). All qPCR reactions were run in duplicate. The average cycle threshold (Ct) values of *CYP9BU1* and *CYP9BU2* were normalized to the geometric mean of the three housekeepi 1g genes using the comparative quantification method (2<sup>- $\Delta\Delta$ Ct</sup>). The  $\Delta$ Ct value of each treatment grc up v as subtracted by the  $\Delta$ Ct value of the MIX pollen not exposed to thiacloprid (water-acetone-co. trol, to yield  $\Delta\Delta$ Ct.

#### 2.6. Statistical analysis

#### 2.6.1. General considerations

All statistical analyses were performed in R ver ion 4.3.2. (R Development Core Team 2023). Model assumptions of normality and homosceda. ticity of residuals, as well as homogeneity of variances in different treatment groups were visualle issessed (Zuur et al. 2009). Statistical inferences were calculated via type II ANOVA using the *car* package for linear models (LMs) and via likelihood ratio tests for generalised linear models (GLSs). Type II ANOVAs were computed manually for generalised least-squares fitted linear models (GLSs) by fitting all sequences of the explanatory variables. A few larvae fell from the pollen or visions during development. These larvae were excluded from all analyses. Exact sample sizes for each model and treatment condition can be found in Supplementary Tables 2 and 3. As the sex of bees that died during larval development could not be determined, all bees (female and male bees) were included in the analysis on survival. Only female bees that reached the cocoon stage (independently of whether they emerged as adults or not) were included in the analyses of development time, pollen consumption, pollen efficacy and cocoon weight, as these response variables may be influenced by the sex of the developing bee. Bees that did not successfully complete metamorphosis inside the cocoon were excluded from the analysis of cocoon weight and pollen efficacy. Separate analyses of males were not performed due to low male sample sizes (see 2.4, above).

Statistical analyses of development time, pollen efficacy and cocoon weight were done using LMs or GLSs, whereas for pollen consumption (pollen fully consumed or not) and survival (emerged as adult or not) GLMs with binomial error distributions were used. Unfortunately, the absolute amount of consumed pollen could not be analysed due to a highly skewed distribution of the data. For development time, pollen efficacy and cocoon weight LMs were fitted initially, however, in several cases, the assumptions of homogeneous residual variances among treatment groups were not met (Zuur et al. 2009). Therefore, GLSs (package *nlme*; Pinheiro et al. 2020) including a function to account for nonhomogeneous variances among treatment groups (*varIdent*) were fitted instead (see detailed model descriptions below). Moreover, for a set of models, it was necessary to use a boxcox transformation of the response variable (using the function *powerTransform* i... the package *car*; Fox and Weisberg 2019) in order to meet model assumptions (see below).

#### 2.6.2. Effects of nutrition

In a first step, to test the effects of the different nutrition types on development time, pollen consumption, pollen efficacy, cocoon weight and sur ival independently from potential interactive effects with pesticides (research questions 1 and 2) LMs, GLMs or GLSs were used, exclusively with data of control bees that were not exposed to pesticides. The models included nutrition type (factor with four levels; MIX, C, S, BQ), a two-level factor accounting for the type of control treatment (water-only-control or water-acetone-control), as well as a covariate accounting for the day on which each egg was collected (egg collection day; continuous) as explanatory (fixed) variables. Egg collection day was included to control for any potential systematic effect of the ongoing season on the laid eggs. For example, changes in resource availability in the surrounding landscape during the season might potentially affect the investment of mothers (Bosch 2008) or the timing of egg laying might affect egg sizes (Maeta and Sugiura 1990). No significant effect of control treatment type (i.e., a difference between water-only and water-acetone controls) was found on any of the analysed response variables, which was therefore not included in the final models. Similarly, egg collection day was only included in the final models if it had

a significant effect ( $P \le 0.05$ ). Thus, the resulting models were in the form: response variable ~ nutrition type (+ egg collection day).

The effect of nutrition type on O. bicornis development time (time from hatching from the egg as larva until initiation of cocoon spinning) was analysed with an LM; the effect on pollen consumption (i.e., the probability of bees to consume the full provision of 400 mg) was analysed with a GLM with binomial error distribution (1: a bee consumed the entire pollen provision, 0: there was leftover pollen); the effects on cocoon weight and pollen efficacy (rate at which consumed pollen is transformed into bee body weight, i.e., cocoon weight divided by the amount of pollen consum d) (both response variables boxcox transformed) were analysed with GLSs accounting for unequal v trian ses across different nutrition types (function varIdent) and the effect on O. bicornis survival www analysed with a GLM with binomial error distribution (1: bee survived and emerged as adult, 0: bee fied during development or did not emerge as adult). To test for significant differences among the one levels of the nutrition treatment (C, S, BQ, MIX), pairwise comparisons using Tukey's ASF tests were made using the package emmeans (Lenth 2021). Moreover, to address research que tion 2, i.e., whether bees feeding on the MIX nutrition type (higher pollen diversity) performed bett r. h; in the average of the low diversity nutrition bees (C, S, and BQ), a contrast was specified for  $c_{2}$ , parison according to a linear hypothesis as follows: (nutrition C + nutrition S + nutrition BQ) / 3 = 0 MIX nutrition was the reference level in the model) using the *glht* function of the *multcomp* p icka; e (Hothorn et al. 2008).

#### 2.6.3. Interactions between pesticides and nutrition

Interactive effects of pesticides with nutrition type (research question 3) on development time, pollen consumption, pollen efficacy, cocoon weight and survival were analysed using (G)LMs or GLSs. Each model (separate models for each pesticide and its interaction with nutrition) contained pesticide concentration (factor with three levels: high pesticide concentration, low pesticide concentration, water-acetone-control), nutrition type (factor: four levels: C, S, BQ, MIX) and their interaction as explanatory variables and egg collection day (numeric) as covariate (this covariate was not included in the final model if it was not statistically significant ( $P \le 0.05$ )). Only the water-acetone-control was used as

control treatment as pesticides were solved in acetone and explorative analyses showed no difference between the water-acetone and water-only control treatments in any of the analysed response variables. The resulting model structure was therefore: response variable ~ pesticide concentration \* nutrition type (+ egg collection day).

Development time was analysed using LMs or GLSs (in case variance was non-homogeneous in the different nutrition types) (LMs: flupyradifurone, thiacloprid, azoxystrobin (the latter two boxcoxtransformed); GLSs: sulfoxaflor, tebuconazole (boxcox-transforme i for tebuconazole)). The pollen efficacy was analysed either using LMs (for sulfoxaflor and azo, ystrobin), LMs with boxcox transformation of the response variable (for flupyradifurone) or 3LS accounting for unequal variances in nutrition types and boxcox transformation (for thiacloprid, and cobuconazole). The cocoon weight was analysed using GLSs accounting for different variances in the individual nutrition types and, when necessary, boxcox transformation (all pesticides ex ep. fc. sulfoxaflor). The response variables survival of O. bicornis and pollen consumption were analysed with GLMs with binomial error distribution (see above). Mean bias-reduced models using method ="brglmFit" (package brglm2; Kosmidis 2021) were used for binomial models due to complete separation. The gene expression levels (log-transformed) were analysed separately for both 3c. is using an LM (CYP9BU1) or a GLS (CYP9BU2, to correct for non-homogeneous variances amo. r nutrition types) including thiacloprid concentration and nutrition type and their interaction  $a \in ext$  lanatory variables. The sample sizes for the different treatment groups were as follows: water-aceto re-control (MIX: 13, C: 16, BQ: 15), high thiacloprid concentration (MIX: 15, C: 13, BQ: 15).

To test for significant differences among treatment levels of a pesticide, overall and within each nutrition type, Tukeys' HSD post-hoc tests were used. Additionally, to address research question 6), a contrast was specified to test the effect of the high pesticide concentration in the MIX nutrition type (higher diversity pollen) compared to the average high pesticide concentration effect in the lower diversity nutrition types (C, S, BQ) using the *glht* function in the package *multcomp*. The tested linear hypothesis was as follows: (high pesticide concentration  $\times$  nutrition C + high pesticide concentration  $\times$  nutrition S

+ high concentration pesticide  $\times$  nutrition BQ) / 3 = 0 (MIX nutrition was the reference level in the models).

#### **3. Results**

#### **3.1. Effects of nutrition**

The type of pollen provision (nutrition type) affected the development time of O. bicornis (Fig. 1a), the probability of consuming the entire amount of provisioned pollen (Fig. 1b), pollen efficacy (Fig. 1c), and cocoon weight at pre-wintering (Fig. 1d; all variables P < 0.001), then larvae were not exposed to any pesticide (water-only-control and water-acetone-control). Larve f d with low diversity nutrition types (*Cistus, Salix, Brassicaceae/Quercus*) developed on av ra<sub>5</sub>~ 2.8% slower than larvae reared on the higher diversity (MIX) nutrition (t = 5.08, P < 0.001). The probability of bees to consume the whole pollen provision was highest when they were feeding on *Cistus* pollen, slightly lower on MIX pollen, and strongly reduced on Salix or Brassicaceae/Oue ccus pollen. Bees feeding on MIX pollen were more likely to consume the whole pollen provision "an the average of bees feeding on the lower diversity nutrition types (z = -2.38, P = 0.018). Bees ind with *Cistus* pollen provisions showed the lowest pollen efficacy, which was 13.2% lower than the efficacy of bees fed with the MIX pollen (t = 30.57, P < 1000.001). Pollen efficacy was on av rage 5.6% higher in bees feeding on MIX pollen compared to bees feeding on the low diversity nutrition types (z = -12.35, P < 0.001), but not significantly different from the best-performing low, "a, 'a' sity nutrition type (Salix). Cocoons were on average 9.6% heavier when larvae were reared on ML nutrition compared to the average of the low diversity nutrition types (z = -14.71, P < 0.001), and 5.9% heavier than the cocoons of the best-performing low diversity nutrition type (Salix, t = 6.95, P < 0.001). The survival probability of O. bicornis larvae reared on pollen provisions not containing pesticides was on average over 90%, and not significantly affected by nutrition type (Fig. 1e). The statistical results are listed in Supplementary Table 4.



**Fig. 1: Nutrition effects.** Estimated mean ( $\pm$  95% confidence intervals) **a**) development time (hatching until cocoon spinning, day.), **b**) pollen consumption (proportion of fully consumed pollen provisions), **c**) pollen efficacy (cocoon weight divided by amount of pollen consumed, mg/mg), **d**) cocoon weight at pre-wintering (mg), and **e**) survival probability until hatching from the cocoon of *O. bicornis* across different nutrition (pollen provision) types: MIX: mixture of *Cistus, Salix* and *Brassicaceae/Quercus* pollen, C: *Cistus* pollen, S: *Salix* pollen, BQ: *Brassicaceae/Quercus* pollen. Raw data is shown as grey dots for development time, pollen efficacy and cocoon weight. Dashed grey lines indicate the average of the low diversity (C, S, BQ) nutrition types. Significant differences ( $P \le 0.05$ ) between nutrition types are indicated with different letters, n.s.: not significant. Only *O. bicornis* not exposed to pesticides (water-only-control, water-acetone-control) were included in these analyses.

#### 3.2. Effects of pesticides and their interaction with nutrition

#### 3.2.1. Sulfoxaflor

Exposure to sulfoxaflor negatively affected development time, pollen consumption and efficacy, cocoon weight at pre-wintering and survival probability until adult emergence of developing O. bicornis (all variables P < 0.001; Fig. 2; Supplementary Figs. 4-8; Supplementary Table 5). The high sulfoxaflor concentration elongated mean development time by 16.7% when compared to the control (t = -11.88, P < 0.001) or 15.8% when compared to the low sulfoxaflor concentrat. on (t = -11.16, P < 0.001). When pollen contained the high concentration of sulfoxaflor, bees were less likely to consume the entire pollen provision compared to control pollen (z = 2.84, P = 0.013) or 'omt ared to pollen containing the low sulfoxaflor concentration (z = 3.86, P < 0.001). Further, which compared to the control and the low sulfoxaflor treatments, respectively, high sulfoxaflor exposure reduced pollen efficacy by 11.8% (t = 10.08, P < 0.001) and 13.7% (t = 11.96, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001), a. "Left as cocoon weight by 24.7% (t = 10.94, P < 0.001]. 0.001) and 26.3% (t = 12.17, P < 0.001) and su vival probability by 33.3% (z = 4.28, P < 0.001) and 31.9% (z = 4.29, P < 0.001). Negative effects of sulfoxaflor were dependent on the nutrition type in the case of pollen efficacy (interaction P = 0.03 /) and cocoon weight (interaction P < 0.001; Fig. 3). The MIX nutrition type did, however, not mitigate the effect of the high sulfoxaflor concentration in these endpoints when compared to the verage effect of the high sulfoxaflor concentration in the lower diversity nutrition types (F. 3. 4)

#### 3.2.2. Flupyradifurone

Exposure to flupyradifurone elongated development time and reduced pollen efficacy and cocoon weight (all variables P < 0.001), but did not significantly affect pollen consumption or survival probability (Fig. 2, Supplementary Figs. 4-8, Supplementary Table 5). The low flupyradifurone concentration increased the development time by 3.2% compared to the control (t = -2.44, P = 0.040), while the high concentration increased the development time by 8.8% compared to the control (t = -6.52, P < 0.001) and by 5.4% compared to the low concentration (t = -4.20, P < 0.001). High flupyradifurone exposure reduced pollen efficacy by 2.6% (t = 2.91, P = 0.012) and 3.2% (t = 3.71, P < 0.001).

0.001) and cocoon weight by 5% (t = 3.68, P < 0.001) and 3.4% (t = 2.45, P = 0.041) compared to the control and the low flupyradifurone concentration, respectively. The effect of flupyradifurone differed depending on the nutrition type for cocoon weight (interaction P < 0.001) and pollen efficacy (interaction P = 0.011; Fig. 3), but the effect of the high flupyradifurone concentration was not mitigated by the MIX nutrition in these endpoints (Fig. 4).

#### 3.2.3. Thiacloprid

Exposure to thiacloprid elongated development time (P < 0.001), but did not significantly affect pollen consumption, pollen efficacy, cocoon weight or survival until adult emorgence (Fig. 2, Supplementary Figs. 4-8, Supplementary Table 5). The high concentration of 'hiac'oprid increased the development time by 4% compared to the control (t = -3.52, P = 0.002)  $e_{1.5}$  by 4.4% compared to the low thiacloprid concentration (t = -3.83, P = 0.001). We did not find interaction.<sup>5</sup> of thiacloprid and nutrition type on any of the analysed endpoints.

#### 3.2.4. Azoxystrobin

Azoxystrobin exposure affected pollen ef.ic acy (P = 0.014), cocoon weight (P < 0.001) and survival probability until adult emergence (P = 0.021), but not development time or pollen consumption (Fig. 2, Supplementary Figs. 4-8, Supplementary Table 5). Pollen efficacy was 3.4% higher in bees treated with the low azoxystrobin concentration compared to those of the control group (t = -2.99, P = 0.009). For cocoon weight, we found no significant negative effects of the high and low concentrations of azoxystrobin compared to the control. Even though the azoxystrobin treatment had an effect on survival, no significant differences between the two azoxystrobin concentrations and the control group were found. There was, however, a marginally significant difference between the low and the high azoxystrobin concentration (z = 2.34, P = 0.051), indicating that the high concentration tended to reduce the survival probability of bees on average by 11.5% compared to the low concentration (Supplementary Fig. 8). Azoxystrobin exposure and nutrition type did not interact in any of the analysed endpoints.

#### 3.2.5. Tebuconazole

Tebuconazole exposure affected pollen efficacy (P = 0.038), but not pollen consumption, cocoon weight or survival probability until adult emergence (Fig. 2, Supplementary Figs. 4-8, Supplementary Table 5). For pollen efficacy, however, no pairwise differences between the two tebuconazole concentrations and the control were found. The effect of tebuconazole exposure on development time varied depending on the nutrition type (interaction P = 0.001, Fig. 3). The effect of the high tebuconazole concentration on development time differed between the MIX and the lower-diversity nutrition types (Supplementary Fig. 9).



**Fig. 2: Pesticide main effects**: Estimated mean ( $\pm$  95% confidence intervals; averaged across the four nutrition types) development time (hatching until cocoon spinning, days), pollen consumption (proportion of fully consumed pollen provisions), pollen efficacy (cocoon weight divided by amount of

pollen consumed, mg/mg), cocoon weight at pre-wintering (mg) and survival until adult emergence of *O. bicornis* across different pesticide treatments: *black*: control, *orange*: low pesticide concentration, *blue*: high pesticide concentration of sulfoxaflor (low: 0.3 / high: 3 mg/kg (SUL)), flupyradifurone (4 / 40 mg/kg (FLU)), thiacloprid (0.075 / 0.75 mg/kg (THI)), azoxystrobin (0.19 / 1.9 mg/kg (AZO)) and tebuconazole (0.05 / 0.5 mg/kg (TEB)). Significant pairwise differences ( $P \le 0.05$ ) between pesticide concentrations (averaged across nutrition types) are indicated with different letters, n.s.: not significant.



**Fig. 3:** Interactive effects between pesticides and nutrition types. Effects of different pesticide concentrations (*black*: control, *orange*: low conc., *blue*: high conc.) and nutrition types (MIX: mixture of *Cistus*, *Salix* and *Brassicaceae/Quercus* pollen, C: *Cistus* pollen, S: *Salix* pollen, BQ: *Brassicaceae/Quercus* pollen) on *O. bicornis* pollen efficacy (cocoon weight divided by amount of pollen consumed, mg/mg), cocoon weight at pre-wintering (mg) and development time (hatching until

cocoon spinning, days). Pesticide concentrations: sulfoxaflor (SUL) low: 0.3 mg/kg, high: 3 mg/kg; flupyradifurone (FLU) low: 4 mg/kg, high 40 mg/kg; tebuconazole (TEB) low: 0.05 mg/kg, high: 0.5 mg/kg. Bars depict estimated means and 95% confidence intervals. Raw data points are indicated as dots. Different letters indicate significant pairwise differences ( $P \le 0.05$ ; Tukey's HSD) between pesticide concentrations *within* nutrition types; no letters indicate that there were no significant differences. Only models with statistically significant interactions between pesticide concentration and nutrition type ( $P \le 0.05$ ) are shown.



Fig. 4: Effect size ( $\pm$  SE) of the high pesticide concentration vs. water-acetone-control in higher diversity (MIX) vs. lower diversity (mean of C, S, BQ) nutrition types on pollen efficacy (*left*, mg/mg) and cocoon weight (*right*, mg). High pesticide concentrations are SUL: 3 mg/kg (*top panel*) and FLU: 40 mg/kg (*bottom panel*). Nutrition types: MIX: mixture of *Cistus*, *Salix* and *Brassicaceae/Quercus* pollen, C: *Cistus* pollen, S: *Salix* pollen, BQ: *Brassicaceae/Quercus* pollen. Values of FLU plots are still on the boxcox scale. \*: Effect sizes are significantly different ( $P \le 0.05$ ).

Only models with statistically significant interactions between pesticide concentration and nutrition type  $(P \le 0.05)$  are shown.

#### 3.3. Expression of detoxification genes

We found that both genes (*CYP9BU1* and *CYP9BU2*) were differentially expressed depending on pollen nutrition type (for both genes P < 0.001; Fig. 5a, b, Supplementary Table 6). The expression of both *CYP9BU1* and *CYP9BU2* was significantly lower in larvae feeding on *Cistus* pollen compared to those feeding on *Brassicaceae/Quercus* (*CYP9BU1*: t = -6.87, P < 0.001; *CYP9BU2*: t = -5.81, P < 0.001) or MIX pollen (*CYP9BU1*: t = 4.25, P < 0.001; *CYP9BU2*: t = 5.99, F < 0.001). *CYPBU2* expression was affected by thiacloprid (P < 0.001; Fig. 5b): expression was on . verage 27.7% lower in larvae exposed to the high thiacloprid concentration when compared to the curred group (t = 3.10, P = 0.003). We did not find significant interactions of thiacloprid exposure and nutrition type on the expression of the two genes.



**Fig. 5: Gene expression of a)** *CYP9BU1* and b) *CYP9BU2* detoxification genes. Estimated mean (± 95% confidence intervals) gene expression expressed as fold change relative to the mean expression level in the MIX nutrition control (no thiacloprid exposure) group of bees. *Black*: control (no thiacloprid exposure), *blue*: exposure to 0.75 mg/kg thiacloprid (high concentration). Nutrition types: MIX: mixture

of *Cistus*, *Salix* and *Brassicaceae/Quercus* pollen, C: *Cistus* pollen, BQ: *Brassicaceae/Quercus* pollen. Raw data is shown as dots. Significant differences ( $P \le 0.05$ ; Tukey's HSD) between pesticide treatments *within* nutrition types are indicated with different letters; no letters indicate that there were no significant differences.

## 4. Discussion

We experimentally investigated the single and interactive effects of distinct pollen nutrition types and exposure to three nicotinic acetylcholine receptor (nAChR) binding ... ecticides and two fungicides on the development and mortality of the solitary bee *O. bicornis*. Our f ndir, 3s demonstrate a clear positive effect of increasing the plant diversity in pollen diet: larvae leve oped faster and transformed the ingested pollen more efficiently into body mass (pollen efficacy), resulting in an increased weight after completion of metamorphosis (cocoon weight). Moreov *r*, the higher of the two tested field-realistic concentrations of all three insecticides thiacle product sulfoxaflor and flupyradifurone, and low concentrations of flupyradifrone had various adverse sub-lethal effects on *O. bicornis* development. High exposure of sulfoxaflor had also lected impacts and decreased survival by 33%. We found no evidence for significant negative effect, of the fungicides tebuconazole and azoxystrobin. Some negative pesticide impacts were non-bated by the type of pollen nutrition, but not in consistent ways, and we found no support for the hypothesis that such negative impacts are mitigated by a more diverse pollen diet.

#### 4.1. Effects of pollen nutrition composition

The pollen nutrition type did not cause substantial difference in mortality of bees not exposed to pesticides with on average more than 90% completing metamorphosis and emerging as adults. This suggests that nutritional properties of the different pollen types, despite some considerable variation (Supplementary Table 1), were still in the tolerance range for *O. bicornis*. This is in line with findings that e.g., even strongly varying protein to lipid ratios had no significant impact on the mortality of developing honeybees (Crone and Grozinger 2021). Nevertheless, nutrition type strongly affected various measured sub-lethal endpoints of *O. bicornis* development and performance, such as

development time, pollen consumption, pollen efficacy and cocoon weight before diapause. For O. bicornis, a longer development time is disadvantageous as the larval stage is particularly vulnerable to environmental stresses, parasitism or infestation by pathogens (Eeraerts et al. 2020). In contrast, bees profit from large body sizes through fitness benefits: positive relationships between body size of female Osmia and offspring provisioning performance, longevity and fecundity have been demonstrated in a series of studies (e.g., Bosch and Kemp 2004, Bosch and Vicens 2006, Seidelmann et al. 2010). Importantly, for these potentially fitness relevant measures, bees feeding on the more diverse pollen mixture performed better than expected based on the average of becs feeding on the individual lower diversity constituents of the mixture. Our results for solitary O. bice rms bees are in a line with findings from studies of developing bumblebee offspring that reached hig test, ody weight on mixed pollen, even though it was not the pollen with the highest protein content in the experiment (Tasei and Aupinel 2008). When fed with a mixture of three pollen types, bumblebee', pollen efficacy was also significantly higher than expected from the average efficacy across the formet mono-floral types (Moerman et al. 2017). Nonetheless, in our study, the efficacy of O. 'ico' nis feeding on Salix pollen was similarly high as those feeding on the pollen mixture, suggesting that a higher pollen diversity is not necessarily always better than a lower diversity nutrition, given t<sup>1</sup> at specific nutrients are available in adequate amount and composition (Radmacher and Stron. 2010, Moerman et al. 2017). Yet, bees reared on the pollen mixture consumed more of the av. iable pollen provisions and developed into heavier bees that those reared on Salix pollen.

In particular, the availability of protein and amino acids in pollen plays a crucial role in bee development (Roulston and Cane 2002, Vanderplanck et al. 2014, Moerman et al. 2016b). But beyond increasing the amounts of single important nutrients, balancing the availability of different nutrients is important for an optimal diet (Crone and Grozinger 2021). Unbalanced nutrition can have potential negative consequences such as reduced body weight of offspring (Sutcliffe and Plowright 1990, Moerman et al. 2016b, Archer et al. 2021). In our experiment, *Cistus* was selected as an expected "poor" pollen diet as it contained the lowest amounts of proteins and total and essential amino acids (Supplementary Table 1; Barraud et al. 2022), which has been associated with low pollen efficacy, body weight gain and offspring

production in bumblebees (Vanderplanck et al. 2014, Moerman et al. 2016a, Leza et al. 2018). In agreement with these predictions, O. bicornis larvae feeding on Cistus pollen reached the lowest body weight before diapause, mirroring the low pollen efficacy. Beyond the fact that this pollen type was consumed relatively quickly, it was also consumed at higher amounts compared to the other lowdiversity pollen diets, which could reflect attempts of bees to compensate for its relatively poor nutritional quality. The depletion of food stores has been found to be a critical cue for the initiation of the metamorphosis in the solitary bee species O. lignaria (Helm et al. 2017). It is therefore conceivable that cocoon spinning was initiated earlier in bees feeding on *Cistus* pollen due to earlier depletion of the pollen provisions. Interestingly, however, bees feeding on Salix or B ass. aceae/Quercus pollen reached similar weights at the cocoon stage before diapause, dest ite slightly higher protein of Brassicaceae/Quercus pollen compared to Salix pollen. This much be explained by the slightly higher content of total and essential amino acids of *Salix* pollen or b, a more favourable ratio of proteins and lipids. A further interesting finding of our study is ne lighter female cocoon weight (ca. 120 mg) compared to bees reared on pollen collected by fre ely foraging O. bicornis (ca. 150 mg; own unpublished data) or compared to females reared on similar amounts of O. bicornis-collected mono-floral Ranunculus or Sinapis pollen (Sediv, ct al. 2011). This could be due to differences in relative proportions of pollen and nectar and "hus provision consistency of honeybee-collected compared to O. bicornis collected pollen. Also, the absence of microbes in the irradiated pollen used here may have affected bee development () has mpal et al. 2022). Moreover, even though no negative effects of gamma irradiation on nutritional properties of pollen are expected (Yook et al. 1998), the process might lower the palatability for bees (Strange et al. 2023). The survival rate and development time in our experiment were, however, within the range of values observed in other studies of bee development in O. bicornis (Nicholls et al. 2017, Claus et al. 2021).

#### 4.2. Effects of pesticides and their interactions with nutrition

We found negative impacts of all three tested insecticides, particularly of sulfoxaflor and flupyradifurone, on several fitness relevant endpoints of developing *O. bicornis*, but generally only at the higher concentrations reflecting worst-case field-realistic exposure scenarios. All three substances

elongated development time, sulfoxaflor and flupyradifurone also reduced pollen efficacy and cocoon weight, and sulfoxaflor reduced pollen consumption and further had lethal impacts and caused increased mortality. These insecticides interfere with nerve impulse transmission in the central nervous system via modulation of the nicotinic acetylecholine receptors (Matsuda et al. 2001, Cutler et al. 2013, Nauen et al. 2015). Neonicotinoid insecticides have been shown to interfere with locomotion, motor function and cognition in bees (Tomé et al. 2012, Tosi and Nieh 2017) and to affect taste and odour perception (Hesselbach and Scheiner 2018). Thus, it is plausible that the tested nAChR modulating insecticides negatively affected the ability of larvae to consume the pollen provisions (Cresswell et al. 2012), resulting in a slower development, reduced pollen consumption and bod, weight. They might, however, also directly impair development via disturbance of the cholin ergin system in bees (Grünewald and Siefert 2019). Elongations of development time after nec. icolnoid exposure have previously been reported (Abbott et al. 2008), but not all studies have four d such effects (Nicholls et al. 2017, Claus et al. 2021, Mokkapati et al. 2021). Neonicotinoids ca. also reduce the nutritional intake in honeybees, notably even though they apparently prefer nec licotinoid-contaminated nectar (Kessler et al. 2015), suggesting that this is not due a simple reportency effect (Kessler et al. 2015, Thompson et al. 2015, Tosi et al. 2017). The reduced pollen const mpt on might have played an important role in reducing the survival of sulfoxaflor-exposed bees in our experiment. Additionally, exposure might have depleted energy reserves due to energetical, costly detoxification or detrimental changes in the bees' metabolism (du Rand et al. 2017, Tosi t al. 2017). A recent study found, for instance, that exposure of O. bicornis to the neonicotinoid acetam prid at field-realistic levels led to a deprivation of energy reserves and a decrease in the metabolic rate (Mokkapati et al. 2022), while the neonicotinoid imidacloprid has also been reported to similarly lower pollen efficacy in bumblebees, *Bombus terrestris* (Barraud et al. 2020). The use of several nAChR modulating insecticides, including thiacloprid and sulfoxaflor, has recently been restricted in the European Union (European Commission 2020, 2022), but they still remain used in other parts of the world.

Suitable nutrition has been hypothesized to mitigate at least partly negative pesticide impacts on bees (Wahl and Ulm 1983, Ardalani et al. 2021, Crone and Grozinger 2021, Knauer et al. 2022), for example

through enhanced expression of detoxification genes (Johnson et al. 2012, Schmehl et al. 2014). Adequate supply and composition of proteins, lipids and carbohydrates in bees' nutrition can improve detoxification and play a critical role as energy sources for these processes (du Rand et al. 2017). As our findings demonstrate positive effects of more diverse pollen nutrition on larval development (development time, pollen consumption, pollen efficacy and cocoon weight), we expected to find less adverse impacts of pesticides in larvae feeding on more diverse pollen provisions. However, although we found certain interactive effects of the tested pesticides and pollen compositions on fitness relevant endpoints, we did not find support for an overall mitigation effect of negative pesticide effects through increased pollen diversity in the solitary bees' nutrition. Further, e' en , ' mitigation effects of a higher quality nutrition may not be detected as fitness relevant organis nal 1 sponses of bees, analysis of gene expression patterns provide further insights into the underlying p.thways and may help to pick up more subtle influences of nutrition-pesticide interactions in beer (Curta et al. 2022). Indeed, our results show that the expression of the studied CYP9BU1 and CVF 3I 2 genes was strongly affected by the different nutrition treatments, with a lower expression in larvae feeding on Cistus pollen compared to Brassicaceae/Quercus pollen or the polle. mixture. Exposure to thiacloprid, however, did not enhance the expression of these genes. This suggers nat these detoxification genes are constitutively expressed and their expression level is influe, ced by the quality of the provided nutrition, which is also in agreement with previous findings (Peadle et al. 2019). We even found that the expression of CYP9BU2 was negatively affected by hiac oprid exposure. This result has to be interpreted with caution, however, as we also found that exposing to the high concentration of thiacloprid slowed down the development of the bee larvae. Thus, it might be possible that the 12-day old larvae exposed to thiacloprid were smaller at the moment of sampling compared to the non-exposed larvae, potentially explaining the lower levels of gene expression in these bees.

Most studies that have investigated interactive effects of pesticides and nutrition have focused on honeybees (Wahl and Ulm 1983, Di Pasquale et al. 2013, Schmehl et al. 2014, Renzi et al. 2016, Tosi et al. 2017, Tong et al. 2019, Barascou et al. 2021, Crone and Grozinger 2021, Vodovnik et al. 2021) and bumblebees (Dance et al. 2017, Leza et al. 2018, Barraud et al. 2020, Linguadoca et al. 2021,

Wintermantel et al. 2022). For example, studying such interactive effects on bumblebees, B. terrestris, Barraud et al. 2020 found, in agreement with findings of the present study, no evidence that negative impacts of imidacloprid were alleviated when bumblebee workers were fed with a higher quality pollen diet. Dance et al. (2017) tested for interactive effects of mono- vs. poly-floral pollen nutrition and neonicotinoid exposure on bumblebees and did not find a mitigation of negative pesticide effects through a poly-floral diet, despite a main positive effect of poly-floral nutrition. A recent meta-analysis of interactive effects of pesticides with further stressors such as food stress on honeybees and bumblebees concluded that food stress and pesticide exposure generally act additively (Siviter et al. 2021), although also synergistic interactions may occur (Tosi et al. 2017, Lingua oca et al. 2021). These results are generally in line with our findings, but in contrast to our experiments most of the abovementioned studies assessed impacts on adult bee performance (e.g. curvival) rather than larval development, making direct comparisons difficult. Generally, apart iron, differences in experimental designs, comparisons of results obtained from studies on hol. vh es or bumblebees and such on solitary bees should be made with great caution, as these 'yee' roups differ substantially in their life history traits as well as pesticide exposure routes and sen. itivities (Brittain and Potts 2011, Arena and Sgolastra 2014, Sgolastra et al. 2019). For example, ne, at we pesticide and/or nutrition effects on larvae of social bees compared to solitary bees might not a unslate into similarly strong negative consequences for population dynamics, as social bees are predicted to be better capable of buffering losses of individual bees from relatively large colonies (Strau) et al. 2015). For solitary bees, only few studies have investigated interactions of pesticides and nutrition (Cecala et al. 2020, Stuligross and Williams 2020, Klaus et al. 2021, Bednarska et al. 2022, Knauer et al. 2022, Kopit et al. 2022). In a recent field study, Bednarska et al. 2022 found that the diversity of pollen nutrition in larval provisions did not affect the sensitivity of O. bicornis to an insecticide, which agrees with our findings. It is important to note, however, that this result was obtained for newly emerged adults, not for developing bees. On the other hand, they found that pollen diversity was of minor importance for measures such as reproduction or cocoon mass, which contradicts our findings of beneficial effects of a more diverse pollen diet. Synergistic negative impacts of the butenolide insecticide Sivanto with its active ingredient flupyradifurone and food stress on O. bicornis have recently been shown in a semi-field study (Knauer et al. 2022). These negative synergistic

impacts were, however, only found for fitness proxies of adult *O. bicornis* (e.g., foraging performance and reproduction), but not for offspring performance (e.g., survival or weight), indicating that different life stages are differently affected by stressors. Moreover, in contrast to our study that tested for a mitigation through higher diversity pollen in the laboratory, Knauer et al. (2022) investigated nutritionpesticide interactions using multiple foraging plants offering floral resources of varying quality. In another semi-field study, Klaus et al. (2021) reported a potential pesticide mitigation effect of added diverse uncontaminated floral resources to contaminated mono-floral oilseed rape diet on *O. bicornis* offspring development, but this effect may have mainly been driven by reduced pesticide exposure of bees. A possible explanation of the lack of an overall mitigation effect of increased pollen diversity in our study may be that rather than a high pollen diversity *per se*, bal, need composition and availability of important nutritional components is an even more critical factor enhancing bees' capacity to cope with exposure to pesticides (Bukovinszky et al. 2017, Filir jiak  $\neg$  al. 2022).

#### 4.3. Conclusions

Our study demonstrates that an increase in plant diversity in the pollen diet was beneficial for the development of *O. bicornis* bees. These in tings underpin the importance of diversity enhancements that support suitable floral resources in agricultural landscapes, not only to foster more diverse wild pollinator communities with distinct diet preferences, but also to promote the development and healthy populations of diet genera ist tees. Moreover, our findings of adverse effects of the neonicotinoid thiacloprid and of the potential neonicotinoid successors sulfoxaflor and flupyradifurone on several fitness-relevant endpoints, and in the case of sulfoxaflor also including lethal effects on developing solitary bees at high field-realistic exposures scenarios, have important implications for pesticide risk assessment. The nAChR modulating insecticides may therefore negatively affect solitary bee populations even if no negative impact is detected in acute first tier studies on adult bees. This underpins the importance of testing not only impacts on adult solitary bees and acute exposure of pesticides, but to test effects of field-realistic exposure in all life history stages, including the larval development stage. Our results also point to potential carry-over effects to the next generation, should e.g., the reduced weight of females impair their offspring provision performance and fecundity (Stuligross and Williams

2021). We did not find that negative pesticide impacts on developing *O. bicornis* were mitigated by a higher diversity pollen nutrition, but impacts varied across the different pollen nutrition types. Further studies addressing the interactive effects of pesticide exposure and nutritional stress are needed to better understand the interplay of these two key threats and underlying mechanisms to effectively mitigate their consequences on the fitness and population dynamics of wild bees in agroecosystems.

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J. M. Schwarz: Conceptualization, Methodology, Investigation, Data Curation, Formal analysis, Writing - Original Draft, Visualization. A. C. Knauer: Conceptualization, Methodology, Writing -Review & Editing, Visualization. A. Barraud: Methodology, Resources, Writing – Review & Editing. D. Michez: Conceptualization, Methodology, Resources, Writing – Review & Editing. L. Barascou: Methodology, Writing – Review & Editing. V. Dievart: Investigation. C. Alaux: Conceptualization, Methodology, Resources, Writing – Review & Editing. M. Albrecht: Conceptualization, Methodology, Writing – Review & Editing. M. Albrecht: Conceptualization, Methodology, Writing – Review & Editing, Supervision, Funding acquisition.

# Declaration of Generative AI and AI-assisted technologies in the writing process

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## Highlights

- We studied interactions of nutrition and pesticides on solitary bee development
- A higher pollen diversity can reduce development time and increase body weight
- Neurotoxic insecticides, but not fungicides, impaired solitary bee development
- Negative insecticide impacts were not mitigated by a higher pollen diversity
- Detoxification gene expression differed depending on the pollen nutrition type