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BRIEF COMMUNICATION

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The leafcutter bee, *Megachile rotundata*, is more sensitive to *N*-cyanoamidine neonicotinoid and butenolide insecticides than other managed bees

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Recent research has shown that several managed bee species have specific P450 enzymes that are preadapted to confer intrinsic tolerance to some insecticides including certain neonicotinoids. However, the universality of this finding across managed bee pollinators is unclear. Here we show that the alfalfa leafcutter bee, *Megachile rotundata*, lacks such P450 enzymes and is >2,500-fold more sensitive to the neonicotinoid thiacloprid and 170-fold more sensitive to the butenolide insecticide flupyradifurone than other managed bee pollinators. These findings have important implications for the safe use of insecticides in crops where *M. rotundata* is used for pollination, and ensuring that regulatory pesticide risk assessment frameworks are protective of this species.

In common with other insects, bees have evolved biotransformation systems to metabolize many of the natural xenobiotics encountered in their environment into non-toxic compounds[1]. Recent research on three managed bee species, namely western/European honeybees, *Apis mellifera*, buff-tailed bumblebees, *Bombus terrestris* and red mason bees, *Osmia bicornis*, has demonstrated that specific enzymes within these metabolic pathways can also be critically important in determining the sensitivity of bees to insecticides[2–4]. Specifically, cytochrome P450 enzymes belonging to the CYP9Q and CYP9BU subfamilies have been shown to provide protection to certain insecticides from three different classes including *N*-cyanoamidine neonicotinoids[2,3]. This leads to an important question. Is the presence of insecticide-degrading P450 enzymes universal to all bee species, and if not, what are the implications for insecticide sensitivity in species that lack these enzymes? To address this question we used phylogenetic, toxicological and biochemical approaches to characterize the phenotypic and metabolic response of the alfalfa leafcutter bee, *Megachile rotundata*, to select insecticides. *M. rotundata* is one of the most economically important managed solitary bee pollinators worldwide[5]. This species is principally used as a commercial pollinator in alfalfa seed production (*Medicago sativa*), with secondary uses in the pollination of canola/rapeseed (*Brassica napus*) and lowbush blueberries (*Vaccinium angustifolium*)[5–7].

We first asked if the genome of *M. rotundata* encodes P450 enzymes belonging, or closely related to, the CYP9Q and CYP9BU subfamilies that metabolize certain insecticides in other managed bee species. Forty-nine full-length P450 genes were curated from the sequenced genome of *M. rotundata* and named by the P450 nomenclature committee (Supplementary Table 1). Phylogenetic analyses revealed that the cytochrome P450 complement (CYPome) of *M. rotundata*, *A. mellifera*, *B. terrestris* and *O. bicornis* have 1:1 orthologues for all members of the mitochondrial CYP clan, and clans 2 and 4 are almost identical (Supplementary Table 2). The major differences in the CYPomes of the species are found in clan 3, with the CYP9 family showing the greatest interspecies variation (Supplementary Table 2 and Fig. 1a). A maximum likelihood phylogeny of the CYP9 family using P450 sequences derived from the genomes of 12 bee species[2,8] is shown in Fig. 1b. All nine species from the Apidae family have *CYP9Q* genes, with the most basal of the species, *Habropoda laboriosa*, having only one gene member (*CYP9Q9*). *O. bicornis* has two *CYP9BU* genes and *Dufourea novaeangliae* four *CYP9DL* genes that share a common ancestor with the Apidae CYP9Q subfamily, with the relative time for divergence of these subfamilies to the CYP9Q subfamily estimated as 0.32 and 0.72, respectively (Fig. 1b). *M. rotundata* is the only species that has no *CYP9Q* or closely related gene (Fig. 1b).

The finding that *M. rotundata* lacks P450 enzymes belonging, or related to, the CYP9Q subfamily raises important questions about the capacity of this species to metabolize and, by extension tolerate, certain pesticides. To explore this, we examined the sensitivity of *M. rotundata* to the *N*-nitroguanidine neonicotinoid imidacloprid (IMI), the *N*-cyanoamidine neonicotinoid thiacloprid (TCP) and the butenolide insecticide flupyradifurone (FPF). While IMI is highly toxic to honeybees, bumblebees and mason bees, TCP and FPF are classified as practically non-toxic to all three species in acute contact bioassays[2,3,9] (Fig. 2a). In the case of the two neonicotinoids, previous work has demonstrated that this differential toxicity results from marked differences in the efficiency of their metabolism by honeybee and bumblebee P450 enzymes belonging to the CYP9Q subfamily, and the red mason bee P450 enzymes belonging to the CYP9BU subfamily[2,3].

As shown in Fig. 2a all three compounds were highly toxic to *M. rotundata* (contact lethal dose, 50% (LD_{50}) < 2 µg per bee) in acute insecticide bioassays, with uncertainty in the actual endpoints, given the non-standardized nature of the test employed. Notably, this included TCP and FPF, with *M. rotundata* >2,500-fold more sensitive to the former and 170-fold more sensitive to the latter than honeybees, bumblebees and mason bees tested using comparable methodologies. Furthermore, while the three latter species exhibit marked differences (500–2,000-fold) in their sensitivity to TCP and

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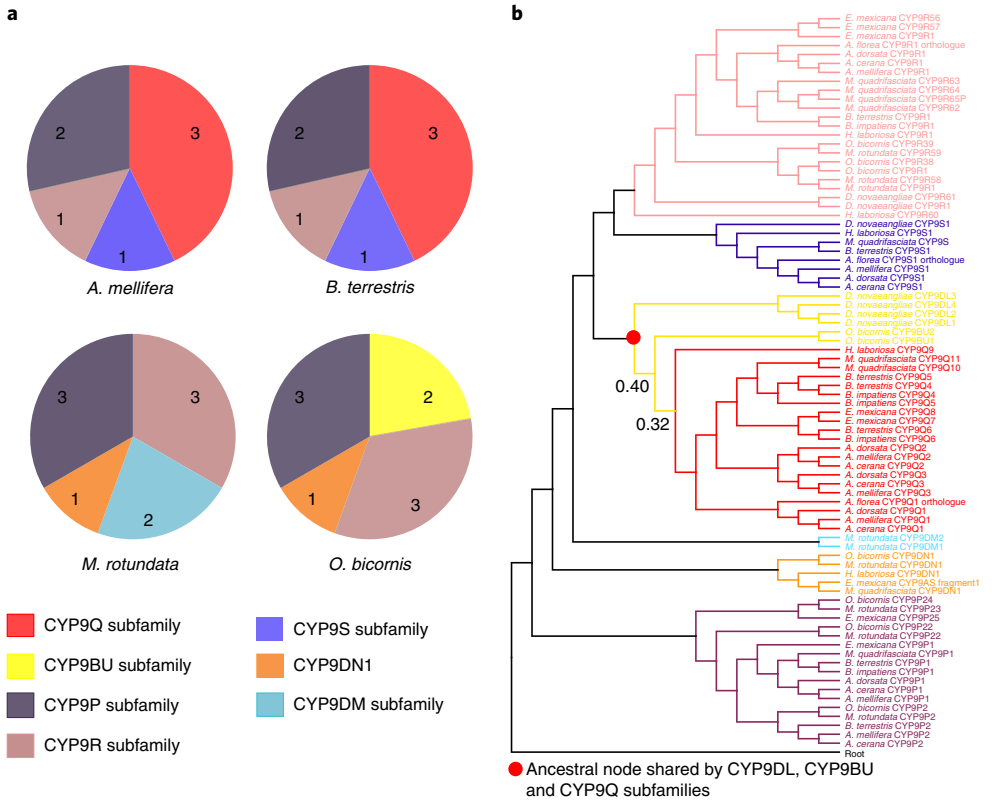

Fig. 1 | Distribution and phylogeny of the *CYP9* family of P450 genes in bee pollinators. a, Distribution of the *CYP9* family across four species of managed bee pollinators. **b**, Maximum likelihood phylogeny of *CYP9* family sequences from 12 species of bee: *A. mellifera*; *A. florea*; *A. dorsata*; *A. cerana*; *B. terrestris*; *B. impatiens*; *D. novaeangliae*; *E. mexicana*; *H. laboriosa*; *M. quadrifasciata*; *M. rotundata*; and *O. bicornis*. The branches show the relative time.

IMI[2,3], only a 15-fold difference is seen in the sensitivity of *M. rotundata* to these two neonicotinoids. These results clearly demonstrate that the intrinsic tolerance of other managed bee pollinators to TCP and FPF is not observed in *M. rotundata*.

One possible explanation for the lack of tolerance of *M. rotundata* to TCP and FPF is an increased affinity of these compounds for the target receptor, the nicotinic acetylcholine receptor (nAChR), of this species relative to other bee pollinators. To explore this, we conducted radioligand binding studies of *M. rotundata* head membrane preparations and examined the displacement of tritiated IMI by unlabelled IMI, TCP and FPF. All three compounds reversibly bind with nanomolar affinity generating similar half maximal inhibitory concentration (IC_{50}) values to those reported previously for honeybees (Fig. 2b). Furthermore, in common with prior studies on other managed bee pollinators[2,3], no significant difference was observed in the specific binding of the three compounds at the receptor (Fig. 2b). Thus, the lack of tolerance of *M. rotundata* to TCP and FPF is not a consequence of an enhanced affinity of these compounds for the *M. rotundata* nAChR relative to other bee species.

To investigate the functional significance of the absence of CYP9Q and CYP9BU P450 enzymes in *M. rotundata* on insecticide metabolism, we examined the capacity of native microsomes (a source of total cytochrome P450 enzymes localized to the endoplasmic reticulum) to metabolize IMI, TCP and FPF. We also included *tau*-fluvalinate (τ-FLV) in this analysis, since it is metabolized by CYP9Q P450 enzymes in honeybees[4], and the alkaloid nicotine (NCT)—a potent natural insecticide also acting on nAChRs. Incubation of microsomal preparations from *M. rotundata* with each compound and analysis of parent compound depletion by liquid chromatography–tandem mass spectrometry (LC–MS/MS) revealed no significant metabolism of any of the four synthetic insecticides (Fig. 2c). In contrast, the alkaloid NCT was significantly and rapidly metabolized by *M. rotundata* microsomes (approximately 40% of parent compound depleted in 1 h; $P < 0.0001$). These findings demonstrate that while the P450 enzymes of this species have the capacity to break down a natural xenobiotic, they appear to lack the capacity to break down synthetic insecticides of different chemical classes that are effectively metabolized by other bee species.

In summary, our data demonstrate that P450 enzymes that are preadapted to detoxify certain insecticides are not ubiquitous across all managed bee species. The absence of P450 enzymes belonging to, or closely related to, the CYP9Q subfamily in *M. rotundata* is correlated with an inability of microsomal P450 enzymes of this species to metabolize these insecticides in vitro and high sensitivity to these compounds in vivo. These results have important practical implications for assessing the compatibility of insecticides in crops where *M. rotundata* provides an important pollination service. Specifically, they demonstrate that direct contact with TCP and FPF poses a greater hazard to this species than other managed bees. Thus, the application method and timing of these insecticides to crops dependent on this species for pollination should be considered. In alfalfa, where *M. rotundata* is a principal commercial pollinator used for seed production, TCP is not registered, whereas FPF has been registered for forage, fodder, hay and straw production. In these uses, alfalfa is harvested before flowering, which effectively mitigates the risk to *M. rotundata*; moreover, alfalfa for seed production is not on the commercial FPF label. Further work is required to establish if *M. rotundata* is also highly sensitive to other insecticides that show low toxicity to other managed bee species. In this regard, we show that microsomal preparations of this species do not metabolize the pyrethroid τ-FLV, suggesting it may also have high acute toxicity to this species. Furthermore, since *Megachile* is one of the largest

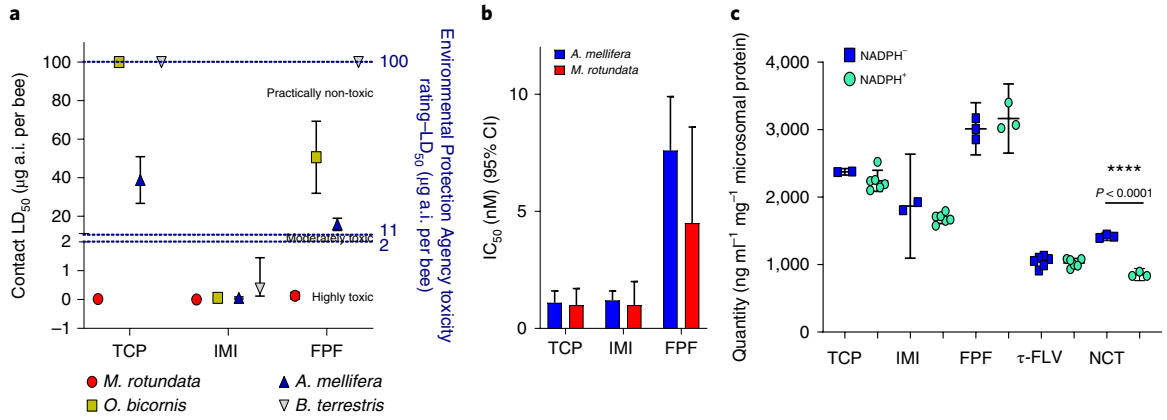

Fig. 2 | Biological and biochemical characterization of the response of *M. rotundata* to select insecticides. a, Contact LD_{50} (48 h) values for topical application of TCP (a.i.), IMI (a.i.) and LD_{50} (72 h) FPF (Sivanto Prime/Sivanto 200 SL) in four managed pollinator species (the error bars indicate the 95% CIs). Sensitivity thresholds are depicted according to the Environmental Protection Agency toxicity ratings[22]. Neonicotinoid data for *A. mellifera, B. terrestris* and *O. bicornis* are taken from refs. [2,3,19]; data for FPF (200 SL) for *A. mellifera* and *B. terrestris* are taken from ref. [17]. **b**, Binding affinity (IC_{50} values) of selected insecticides to nAChR head membrane preparations of *A. mellifera* and *M. rotundata*. Data for *A. mellifera* are taken from ref. [3]. **c**, Metabolism of TCP, IMI, FPF, τ-FLV and NCT by native microsomal preparations of *M. rotundata* as measured by LC-MS/MS (1 h incubation at 30 °C ± NADPH). The error bars indicated 95% CIs. Analysis performed using a Welch's *t*-test (two-tailed; degrees of freedom = 3.581) with significant differences indicate by ****$P < 0.0001$.

genera of bees (1,500 species worldwide), further research is required to establish if other wild species within this genus also lack P450 enzymes that provide protection against certain insecticides. In this regard, our study illustrates the utility of phylogenetic analyses of enzyme superfamilies in combination with targeted functional analyses to predict the capability of bee pollinator species to break down synthetic insecticides, and hence predict their probable sensitivity. Finally, since the use of other managed bee species as a proxy for *M. rotundata* in risk assessment appears to be unreliable for some insecticides, it is important to invest in further bee toxicogenomic research so that crop pollination, managed pollination activities and bee safety are not impaired.

Methods

Phylogenetic analysis. Sequences encoding *M. rotundata* P450 enzymes were identified and assembled using three separate interrogations of the National Center for Biotechnology Information protein database: G1, assembled all annotated cytochrome P450 enzymes (CYPs) from the database; G2, assembled CYP gene clusters or orthologue groups; and G3, assembled the results of a BLASTp (blastp:2.5.0+) search of the *M. rotundata* proteome using annotated *A. mellifera* CYPs as query sequences. The resulting sequences were manually curated and aligned to those of 11 other bee species (*Apis cerana, Apis dorsata, Apis florea, A. mellifera, Bombus impatiens, B. terrestris, D. novaeangliae, Eufriesea mexicana, H. laboriosa, Melipona quadrifasciata* and *O. bicornis*) using CYPcam (camphor hydroxylase from *Pseudomonas putida* (>gi|117297|sp|P00183.2| cytochrome CYP-cam; CPXA_PSEPU)[10] as an outgroup in Geneious v.10.2.3 (Biomatters) using MUSCLE[11] (version 3.5, default settings). Parameters including proportion of variable sites and gamma rate were optimized using amino acid substitution matrices (LG) based on a minimum Bayesian information criterion[12]. Phylogeny was estimated using maximum likelihood (branch lengths in relative time) and Bayesian inference algorithms[13,14].

Acute contact insecticide assays. All acute contact insecticide assays were performed on female bees following the general guidance of the Organisation for Economic Co-operation and Development test no. 214 for honeybees[15], with reference to the International Commission for Plant-Pollinator Relationships solitary bee, acute contact toxicity test protocol[16]. Bioassays on *M. rotundata* using the neonicotinoids TCP and IMI were performed in the UK and compared with data for *A. mellifera*, *B. terrestris* and *O. bicornis* (Fig. 2a) generated in previous studies[2,3] using identical methods and conditions. This allowed robust comparisons to be made between and within these bee species for these compounds. Bioassays on *M. rotundata* and *O. bicornis* using the butenolide insecticide FPF were conducted in Germany and compared with data for *A. mellifera* and *B. terrestris* (Fig. 2a) generated in a previous study[17] using analogous methods and conditions to allow robust comparisons between bee species for this compound. Commercially available *M. rotundata* cocoons were obtained from Canada through Bayer AG Crop Science Division. *O. bicornis* cocoons were obtained from Dr. Schubert Plant Breeding. Cocoons were stored on arrival at 4 °C in constant darkness. To trigger emergence, cocoons were warmed for 24 h (24 ± 2 °C; 55% relative humidity; 16 h light, 8 h dark). Cocoons were then transferred to an incubator (*O. bicornis*: 25 ± 1 °C; 55% relative humidity; 0 h light, 24 h dark; *M. rotundata*: 30 ± 1 °C; 55% relative humidity; 0 h light, 24 h dark) to allow bees to emerge. Males, which are usually first to emerge, were removed and discarded to allow only non-mated females to populate the test cages. Emerged females were removed from the emerging boxes and placed into test cages and kept under test conditions (24 ± 2 °C; 55% relative humidity and 16 h light, 8 h dark) until enough bees were collected to populate a test replicate (60–70). Bees were fed ad libitum with sucrose solution in water with a final concentration of 500 g l^{-1} (50% w/v). Female bees (24–48 h old) were anaesthetized with CO_2 for 10–15 s to allow for the application of insecticide (technical grade IMI or TCP (Sigma-Aldrich) or formulated FPF (commercial product: Sivanto Prime/Sivanto 200 SL)) or insecticide diluent alone (in the case of controls) by topical application with a handheld microapplicator (PB600-1 repeating dispenser; Hamilton). A volume of 1 µl of test substance solution was applied to the dorsal side of the thorax of each bee. Five concentrations of each insecticide were tested (spanning 0.000256 µg active ingredient (a.i.) per bee to 0.05 µg a.i. per bee) with 3 replicates of 10 bees tested for each concentration. Mortality was assessed at 48 h post-application for TCP and IMI and 72 h for FPF. Bioassays with control mortality >10% were excluded from the analysis. The relationship between concentration and mortality was determined using probit analysis with LD_{50} values and their respective 95% confidence interval (CI) values calculated using PoloPlus v.1.0 (Le Ora Software LLC) and SPSS v.24 (IBM Corporation); Supplementary Table 3). Regarding the use of the bioassay guidelines developed for *A. mellifera* in this study, a previous meta-analysis of insecticide sensitivity data generated from 19 bee species (including *M. rotundata*), using different bioassay methods, revealed that the sensitivity ratio of non-honeybee species to the honeybee was <10 for 95% of cases[18].

Receptor binding studies. [^{3}H]IMI (specific activity 1.406 GBq µmol^{-1}) displacement studies were conducted using membrane preparations isolated from frozen (−80 °C) *M. rotundata* heads, following protocols published previously[19]. Briefly, bee heads weighing approximately 10 g were homogenized in 200 ml ice-cold 0.1 M potassium phosphate buffer, pH 7.4, containing 95 mM sucrose using a motor-driven Ultra-Turrax blender. The homogenate was then centrifuged for 10 min at 1,200*g* and the resulting supernatant filtered through five layers of cheesecloth with protein concentration determined using Bradford reagent (Sigma-Aldrich) and BSA as a reference. Assays were performed in a 96-well microtitre plate with bonded GF/C filter membrane (UniFilter-96, GF/C; Packard) and consisted of 200 µl homogenate (0.48 mg protein), 25 µl [^{3}H]IMI (576 pM) and 25 µl competing ligand. The ligand concentrations used ranged from 0.001 to 10,000 nM and were tested in triplicate per competition assay. The assay was started by adding homogenate and incubating for 60 min at room temperature. Bound [^{3}H]IMI was quantified by filtration into a second 96-well filter plate (conditioned with ice-cold 100 mM potassium phosphate buffer, pH 7.4, including

BSA 5 g l^{-1}) using a commercial cell harvester (Brandel). After three washing steps (1 ml each) with buffer, the 96-well filter plates were dried overnight. Each well was then loaded with 25 µl of scintillation cocktail (Microszint-O-Filtercount; Packard) and the plate counted in a TopCount scintillation counter (Packard). Non-specific binding was determined using a final concentration of 10 µM unlabelled IMI. All binding experiments were repeated twice using three replicates per tested ligand concentration. Data were analysed using a 4-parameter logistic non-linear fitting routine (Prism 8 (GraphPad Software)) to calculate the IC_{50} values (concentration of unlabelled ligand displacing 50% of [^{3}H]IMI from its binding site).

Metabolism assays and ultra-performance LC–MS/MS analysis. Microsomes were prepared from approximately 60 adult female *M. rotundata* following a standard protocol of homogenization and differential centrifugation[20]. The protein concentration of microsome preparations was determined using the Bradford reagent and BSA as a reference. Native microsomes (160 µg per well) were incubated for 1 h (with shaking) with insecticide substrates (10 µM) in a total assay volume of 200 µl at 30 ± 1 °C, in the presence or absence of a dihydronicotinamide-adenine dinucleotide phosphate (NADPH) regeneration system. Three replicates were performed for each data point. Samples incubated without NADPH served as controls. The reactions were terminated by adding ice-cold acetonitrile (to 80% final concentration), centrifuged for 10 min at 3,000*g* and the supernatant was subsequently analysed by LC–MS/MS as described previously[21]. LC–MS/MS analysis was performed on an Acquity UPLC (Waters) coupled to an API 4000 mass spectrometer (Sciex) and an Infinity II UHPLC (Agilent Technologies; reverse phase mode) coupled to a QTRAP 6500 mass spectrometer (Sciex) employing electrospray ionization. The recovery rates of parent compounds using microsomal fractions without NADPH were normally close to 100%. Substrate turnover was determined with Prism 8.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The accession numbers of the *M. rotundata* P450 genes analysed in this study are shown in Supplementary Table 1. All other data generated or analysed during the study are included in this published article and its Supplementary Information files.

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Author contributions

C.B. and R.N. conceived and directed the study, A.H., K.B., K.S.S. and J.G. performed the experiments and analysis. N.E., M.Z., M.-T.A., A.N. and C.G. analysed and interpreted the data. A.H., C.B. and R.N. wrote the paper with contributions from all authors.

Competing interests

This study received funding from Bayer AG, a manufacturer of neonicotinoid and butenolide insecticides. N.E., M.Z., M.-T.A., A.N., C.G., J.G. and R.N. are employees of Bayer AG.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41559-019-1011-2.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Microsoft Excel (2011 Version 14.7.2); Video taken using iPhone 4S.
Data analysis	Biomatters Ltd Geneious Version 10.2.3 used for Bayesian inference analysis (default settings: chain length - 1,100,000; subsampling frequency - 200; burn-in length - 100,000; unconstrained branch lengths; rate matrix - LG; rate variation - gamma); MEGA7 used for maximum likelihood analysis; LeOra PoloPlus Version 1.0; IBM SPSS Version 25; GraphPad Prism Version 8.1.0; Microsoft Excel (2011 Version 14.7.2)

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Accession numbers of the M. rotundata P450 genes analysed in this study are shown in Table S1. All other data generated or analysed during this study are included in this published article (and its supplementary information files).

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Study description	Recent research has shown that several managed bee species have specific P450 enzymes that are preadapted to confer intrinsic tolerance to some insecticides, including certain neonicotinoids. Whether these enzymes are ubiquitous across all bee species is unclear. This study uses phylogenetic, toxicological and biochemical approaches to describe the phenotypic and metabolic response of the alfalfa leafcutter bee, Megachile rotundata to select insecticides that are metabolized by other species of managed bee pollinators. Phylogenetic sequences coding for M. rotundata P450 enzymes were curated, aligned and analysed by comparing them to P450 sequences from other bee species. The sensitivity of the species to these insecticides was determined by acute contact toxicology tests (n=10, 3 replicates per insecticide). Radioligand binding studies on M. rotundata head membrane preparations(3 replicates per insecticide) were performed to assess the affinity of the insecticides for the nicotinic acetylcholine receptor. To investigate the global metabolic ability of the species, native M. rotundata microsome incubation LC-MS assays (3 replicates per insecticide) were undertaken.
Research sample	Nucleotide and protein sequences used in the study were obtained from the existing databases on the NCBI. Newly emerged (24-48h) female M. rotundata were used in the acute contact toxicity test. For microsome extraction and radioligand binding studies bees were collected (24h) and directly frozen using liquid N2. The species is used as a commercial, agricultural pollinator and this study therefore represents that economically valuable population. It may also represent other closely related Megachilidae species.
Sampling strategy	Neonicotinoid acute contact toxicity test: sample size (n=10) was chosen with reference to the OECD guideline No. 214 for Apis mellifera.
Data collection	Neonicotinoid acute contact toxicity test: female Megachile rotundata (24-48 hours post emergence) were exposed to a range of doses of test substance dissolved in acetone, by direct application (droplets) to the dorsal side of the thorax using a Hamilton repeating dispenser (PB600-1). The test duration was 96h. Mortality was recorded daily (video recording and pen and paper score) and compared with control values. Three replicates per insecticide performed. All bioassay data was recorded by one individual and subsequently documented in Microsoft Excel (2011 Version 14.7.2).
Timing and spatial scale	Neonicotinoid acute toxicity test: July - September. Replicates were performed weekly. Radioligand competition binding studies: January - February 2019 - three replicates per insecticide performed Native microsome LC-MS assays: nicotine, flupyradifurone, tau-fluvalinate - LC-MS performed November 2017; thiacloprid, imidacloprid LC-MS performed January 2019 - three replicates per insecticide performed.
Data exclusions	Neonicotinoid acute toxicity test: for the test to be valid, the average mortality for the total number of controls must not exceed 10% at the end of the test. No data was excluded. Radioligand competition binding studies: no data excluded. Native microsome LC-MS assays: no data excluded.
Reproducibility	Neonicotinoid acute toxicity test: the standard methodology from the OECD guideline No. 214 for Apis mellifera was followed. Radioligand competition binding studies: standard protocols followed. Native microsome LC-MS assays: standard protocols followed.
Randomization	Neonicotinoid acute toxicity test: holding cages of bees were randomly assigned to the different test doses and controls.
Blinding	Neonicotinoid acute toxicity test: blinding is not required in the OECD guideline No. 214 for Apis mellifera which represent the 'gold standard' model for acute insecticide bioassays of bees.

Did the study involve field work? ☐ Yes ☒ No

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Megachile rotundata is a lower invertebrate and is not classified as a 'Laboratory animal'. Bees were commercially reared and supplied as such.
Wild animals	Study did not involve wild animals
Field-collected samples	Study did not involve samples collected from the field
Ethics oversight	Megachile rotundata is a lower invertebrate and thus study of this species have no ethical requirements.

Note that full information on the approval of the study protocol must also be provided in the manuscript.