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Toxicogenomic studies of the effects of insecticides on the western honey bee (*Apis mellifera*; Hymenoptera: Apidae)

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Marion Zaworra

aus

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Referent: Prof. Dr. Florian M. W. Grundler

Korreferent: Prof. Dr. Christian Borgemeister

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Remember to look up at the stars and not down at your feet. Try to make sense of what you see and wonder about what makes the universe exist. Be curious. And however difficult life may seem, there is always something you can do and succeed at. It matters that you don`t just give up.

Stephen Hawking

To my parents

Zusammenfassung

Die Gesundheit von Bestäuberinsekten und ihr Schutz sind intensiv und kontrovers diskutierte Themen in Wissenschaft, Öffentlichkeit und Politik der letzten Jahre. Ein Kernstück dieser Debatte ist der Einsatz von Insektiziden und ihre potentiellen Effekte auf die westliche Honigbiene *Apis mellifera* L. (Hymenoptera: Apidae) sowie andere Bienenarten.

Insbesondere Insektizide, die zur Klasse der Neonikotinoide gehören, wurden gelegentlich beschuldigt, eine Hauptursache des Rückgangs von Bestäuberinsekten weltweit zu sein. Neonikotinoide sind systemische Insektizide und binden als partielle Agonisten an die postsynaptischen nikotinergen Acetylcholinrezeptoren (nAChR) von Insekten. Sie werden breitflächig zur Bekämpfung von saugenden und bestimmten beißenden Schadinsekten angewendet. Verschiedene Neonikotinoide zeigen abhängig von ihrer Pharmakophorstruktur signifikante Unterschiede in ihrer intrinsischen Toxizität gegenüber *A. mellifera*. Die *N*-cyano-substituierten Moleküle Thiacloprid und Acetamiprid weisen eine zwei bis drei Zehnerpotenzen geringere intrinsische Toxizität auf als *N*-nitro-substituierte Moleküle, beispielsweise Imidacloprid.

Die biochemischen und molekularen Mechanismen, die der Sensitivität von Bienen gegenüber den genannten Neonikotinoiden zugrunde liegen, wurden in dieser Arbeit untersucht.

Radioligandenbindungsstudien, die an Kopfmembranpräparationen der Honigbiene durchgeführt wurden, zeigten, dass Thiacloprid und Imidacloprid mit einer ähnlichen nanomolaren Affinität an ihren molekularen Wirkort binden. Folglich muss der Sensitivitätsunterschied einen anderen Ursprung haben.

Eine Vielzahl von publizierten Studien indizierten, dass Cytochrom P450 Monooxygenasen (P450s) wichtige Enzyme im oxidativen Abbau von Neonikotinoiden in der Honigbiene sind. Daher wurden im Rahmen dieser Arbeit verschiedene *in vivo* und *in vitro* Methoden entwickelt, um die Interaktion der ausgewählten Neonikotinoide mit den P450s von *A. mellifera* mechanistisch zu studieren.

Die in der Vergangenheit beschriebenen Probleme, die mit der Isolation funktioneller Mikrosomen aus abdominalen Präparationen von Honigbienen assoziiert sind, wurden aufgegriffen und durch das simple Entfernen des Giftblase-Stachel-Komplexes vor der Gewebeaufarbeitung gelöst. Eine detaillierte Methode zur Isolation hochfunktioneller Mikrosomen aus Abdomen von Arbeiterinnen wurde beschrieben und der Bienengiftbestandteil Phospholipase A_2 als Faktor, der zur Inaktivierung mikrosomaler P450s vermutlich durch Desintegration der Mikrosomenmembranen führt, identifiziert. Neben der Charakterisierung der metabolischen Kapazität von mikrosomalen P450s mit Hilfe von fluoreszenzbasierten Modellsubstraten, konnte zudem eine signifikant schnellere Detoxifizierung von Thiacloprid im Vergleich zu Imidacloprid *in vitro* gezeigt werden.

Des Weiteren lieferten *in vitro* Studien an funktionell exprimierten P450s der monophyletischen Gruppe 3 wichtige Erkenntnisse über die Rolle einzelner Enzyme im oxidativen Metabolismus der ausgewählten Neonikotinoide. Dabei wurden die drei Enzyme, die der CYP9Q-Subfamilie von *A. mellifera* angehören, im schnellen Abbau von Thiacloprid und Acetamiprid identifiziert. Das Enzym CYP9Q3 stach als effizienter Hauptmetabolisierer von Thiacloprid mittels Hydroxylierung heraus. Gegenüber Imidacloprid zeigten die CYP9Q-Enzyme einen signifikant geringeren Abbau *in vitro* und konnten somit als Schlüsselenzyme, die der Bienensensitivität gegenüber Neonikotinoiden zugrunde liegen, beschrieben werden.

Zudem wurde eine Methode entwickelt, um den *in vivo* Metabolismus und die Pharmakokinetik von [^{14}C]-markierten Neonikotinoiden nach Kontaktapplikation aufzuklären. Dabei zeigte sich, dass die intrinsisch weniger toxischen *N*-Cyanoamidine Thiacloprid und Acetamiprid langsamer durch die Kutikula der Honigbiene penetrieren sowie schneller metabolisiert und ausgeschieden werden, als das intrinsisch hochtoxische *N*-Nitroguanidin Imidacloprid. Unter Anwendung der Methode konnte erstmals der *in vivo* Metabolismus von Thiacloprid in der Honigbiene aufgeklärt werden. Darüber hinaus konnte eine Wissenslücke über das Verhalten von Neonikotinoiden nach Kontaktapplikation geschlossen werden und die Pharmakokinetik als ein weiterer Faktor, welcher der geringeren intrinsischen Toxizität von *N*-cyano-substituierten Neonikotinoiden zugrunde liegt, beschrieben werden.

Die vorliegenden toxikogenomischen Studien betrachten mechanistisch die molekularen und biochemischen Interaktionen von Insektiziden mit der westlichen Honigbiene. Darüber hinaus können die entwickelten Methoden in der Beantwortung von wissenschaftlichen sowie angewandten Fragestellungen für die Evaluation der Bienensicherheit von Pflanzenschutzmitteln Anwendung finden.

Abstract

Pollinator health and safety are among the most intense and controversially discussed topics in science, public and politics of the last years. The use of insecticides and their potential effects on the western honey bee *Apis mellifera* L. (Hymenoptera: Apidae), as well as other bee species, have become a core part of this debate.

In particular, insecticides belonging to the chemical class of neonicotinoids have been occasionally accused to be a key driver in pollinator decline worldwide. Neonicotinoids are systemic insecticides that act as partial agonists of the postsynaptic nicotinic acetylcholine receptor (nAChR) of insects and are widely applied to combat sucking and certain chewing pest species. Different neonicotinoids display differences in their intrinsic toxicity on honey bees with the *N*-cyanoamidines thiacloprid and acetamiprid acting about two to three orders of magnitude less toxic compared to *N*-nitroguanidines such as imidacloprid.

In this thesis, light was shed on the biochemical and molecular mechanisms underlying the honey bee sensitivity towards certain neonicotinoid insecticides.

Radioligand binding assays conducted on head membrane preparations of the honey bee revealed that thiacloprid and imidacloprid display a similar nanomolar binding affinity to postsynaptic nAChRs. In conclusion, the toxicity difference of the compounds does not derive at the molecular target and has to have another origin.

A number of published studies indicated that cytochrome P450 monooxygenases (450s) play a crucial role in the oxidative metabolism of neonicotinoid insecticides in the honey bee. Thus, different *in vivo* and *in vitro* methods were developed to mechanistically assess the interaction of selected neonicotinoid insecticides with honey bee P450s.

First, the previously described problems associated with the isolation of functional microsomes from abdominal preparations of honey bees were approached and solved by the simple removal of the venom gland sting complex prior to tissue homogenization. A detailed method for the isolation of highly active microsomes from whole worker abdomen is outlined and compelling evidence depicted that the bee venom compound phospholipase A_2 is responsible for the inactivation of microsomal P450s, most likely by disintegration of the microsomal membranes.

In addition to the characterization of the detoxification capacity of microsomal P450 with fluorescence based model substrates, the significantly faster P450-driven detoxification of thiacloprid in comparison to imidacloprid was demonstrated *in vitro.*

Important insights on the role of individual P450s belonging to the monophyletic group 3 in the oxidative metabolism of the selected neonicotinoid insecticides were obtained from studies with functional expressed enzymes. The three honey bee P450s belonging to CYP9Q-subfamily have been identified as key enzymes in the rapid metabolism of *N*-cyanoamidine neonicotinoid insecticides with CYP9Q3 highlighted as the particular key enzyme involved in the rapid

detoxification of thiacloprid by hydroxylation *in vitro*. The turnover of imidacloprid by this enzyme family was significantly lower; thus enzymes belonging to CYP9Q-subfamily were identified as molecular determinants mediating bee sensitivity to neonicotinoid insecticides.

A new method was developed in order to elucidate the *in vivo* metabolism and pharmacokinetics of the selected [^{14}C]-labelled neonicotinoids after contact exposure. This study demonstrated that the *N*-cyanoamidines thiacloprid und acetamiprid displaying a lower acute intrinsic toxicity to honey bees showed a slower penetration through the honey bee cuticle in line with a faster metabolization and elimination rate compared to the intrinsically highly toxic *N*-nitroguanidine imidacloprid. Applying this method, the *in vivo* metabolic fate of thiacloprid in honey bees was elucidated for the first time. The study completed a knowledge gap on the contact mode of entry of neonicotinoids and identified the pharmacokinetics as another factor contributing to the lower intrinsic toxicity of thiacloprid and acetamiprid after contact exposure to honey bees.

The outlined toxicogenomic studies provide a mechanistic view on the interaction of honey bees with selected neonicotinoid insecticides. The established biochemical and molecular methods are ready to be applied to address fundamental research questions, as well as applied questions in the bee safety evaluation of crop protection products.

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List of Publications

Chapter 2

Zaworra, M., Nauen, R., 2019. New approaches to old problems: Removal of phospholipase A_2 results in highly active microsomal membranes from the honey bee, *Apis mellifera*. Pestic Biochem Physiol. 161: 68-76. https://doi.org/10.1016/j.pestbp.2019.04.014

Chapter 3

Manjon, C*., Troczka, B.J.*, Zaworra, M.*, Beadle, K., Randall, E., Hertlein, G., Singh, K.S., Zimmer, C.T., Homen, R.A., Lueke, B., Reid, R., Kor, L., Kohler, M., Benting, J., Williamson, M.S., Davies, T.G.E., Field, L.M., Bass, C., Nauen, R., 2018. Unravelling the Molecular Determinants of Bee Sensitivity to Neonicotinoid Insecticides. Curr Biol. 28(7): 1137-1143.e5. https://doi.org/10.1016/j.cub.2018.02.045

* These authors contributed equally

Chapter 4

Zaworra, M., Koehler, H., Schneider, J., Lagojda, A., Nauen, R., 2019. Pharmacokinetics of Three Neonicotinoid Insecticides upon Contact Exposure in the Western Honey Bee, *Apis mellifera*. Chem Res Toxicol. 32(1): 35-37. https://doi.org/10.1021/acs.chemrestox.8b00315

List of Abbreviations

%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microliter
µM	Micromolar
6-CNA	6-Chloronicotinic acid
a.i.	Active ingredient
ABT	1-aminobenzotriazole
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACT	Acetamiprid
AD	Antagonistic binding domain
TD	Transmembrane domain
ADME	Absorption, distribution, metabolism and excretion
ATP	Adenosine triphosphate
BFC	7-benzyloxy-4-trifluoromethyl coumarin
BOMFC	7-benzyloxymethoxy-4-trifluoromethyl coumarin
BOMR	7-benzyloxymethoxyresorufin
BOR	7-benzyloxyresorufin
BSA	Bovine serum albumin
CCE	Carboxylesterase
CD	Cytoplasmic domain
cDNA	Complementary DNA
CL	Confidence interval
CO	Carbon monoxide
CO_2	Carbon dioxide
CYP	Cytochrome P450 monooxygenase
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DWV	Deformed wing virus
e.g.	Example given
EBI	Ergosterol biosynthesis inhibitor
EC	7-ethoxy coumarin
EDTA	Ethylenediaminetetraacetic acid
EFC	7-ethoxy-4-trifluoromethyl coumarin
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
EPPO	European and Mediterranean Plant Protection Organization
ER	7-ethoxyresorufin
ER	Endoplasmic reticulum
ESI-MS	Electrospray ionization mass spectroscopy
et al.	*et alia* (and others)
EU	European Union
Fig.	Figure

FPLC	Fast protein liquid chromatography
g	Gram
g	relative centrifugation force
GLP	Good laboratory practice
GSH	Glutathione
GST	Glutathione S-transferase
h	Hour(s)
H_2O	Water
ha	Hectare
HPLC	High performance liquid chromatography
IMD	Imidacloprid
IRAC	Insecticide Resistance Action Committee
IUPAC	International Union of Pure and Applied Chemistry
kDa	Kilo Dalton
kdr	Knockdown resistance
KH_2PO_4	Monopotassium phosphate
Km	Michaelis constant
L	Liter
LC-MS/MS	Liquid chromatography coupled to mass spectroscopy
LCS	Liquid scintillation counting
LD_{50}	Median lethal dose, dose at which 50 % of the individuals tested died
Log P_{ow}	octanol-water partition coefficient
M	Molar
Mbq	Mega becquerel
MC	7-methoxy coumarin
MeCN	Acetonitrile
MFC	7-methoxy-4-trifluoromethyl coumarin
mg	Milligram
min	Minutes
ml	Milliliter
mM	Millimolar
MOBFC	7-p-methoxy-benzyloxy-4-trifluoro coumarin
MR	7-methoxyresorufin
MS	Mass spectroscopy
Na_2HPO_4	Disodium phosphate
nAChR	Nicotinic acetylcholine receptor
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide hydrogen phosphate
nM	Nanomolar
O_2	Oxygen
OECD	Organization for Economic Co-operation and Development
OOMR	Octyloxymethoxyresorufin
P450(s)	Cytochrome P450 monooxygenase(s)
PBO	Piperonyl butoxide
PC	7-n-pentoxy coumarin
PCR	Polymerase chain reaction
pH	Negative logarithm of the activity of solvated hydronium ions
PLA_2	Phospholipase A_2

ppm	Parts per million
PR	7-n-pentoxyresorufin
RFU	Relative Fluorescence Unit
RNA	Ribonucleic acid
SD	Standard deviation
SDS-page	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error
SEM	Standard error mean
TCP	Thiacloprid
U	Enzyme unit
US$	United States Dollar
UV	Ultraviolet
V	Volt
v/v	Volume per volume
v/w	Weight per volume
*V*max	Maximum velocity
VS	Venom gland sting complex

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Chapter 1

Introduction

Over the last decade pollinator health related issues have become intensively discussed topics in science, politics and the media. The health of honey bees, *Apis mellifera* L. (Hymenoptera: Apidae), has received particular attention as it is affected by numerous factors. The infestation with pathogens, changes of agricultural landscape structures and crop diversity, as well as the effects of crop protection products have been addressed in a considerable number of publications as potential factors affecting pollinator health worldwide.

The western honey bee *A. mellifera* is a well-studied bee species in terms of physiological and behavioral traits and is the predominately used bee species to assess the toxicity of crop protection products mandatory to meet the regulatory requirements to approve and register these products in the European Union (EU). While the acute intrinsic toxicity of insecticides to honey bees after oral or contact exposure has been thoroughly tested for all commercially available compounds, less is known about the general detoxification capacity of the honey bee towards exogenous compounds, such as insecticides. However, a broad knowledge and detailed understanding about the physiology and detoxification capacity of honey bees is required in order to design bee-friendly insecticides in a targeted and efficient way.

In 2002, the debate on honey bee health became advert in Germany when increased overwintering colony mortality rates were recorded (Genersch *et al.* 2010a). A completely different aspect related to bee mortality came into the focus of the public debate in spring 2008 when the neonicotinoid insecticide clothianidin abraded from improperly coated corn seeds during drilling in the Upper Rhine Valley (Southwestern Germany) (Pistorius *et al.* 2009, Nikolakis *et al.* 2009). The resulting dust emissions were distributed in the landscape and contaminated bee-attractive crops and weeds in the surrounding area which in turn harmed approximately 11,500 bee hives (Pistorius *et al.* 2009, Nikolakis *et al.* 2009).

Neonicotinoids are systemic insecticides targeting the nicotinic acetylcholine receptors (nAChR) of insects as partial agonists and are widely applied to control a broad spectrum of sucking and certain chewing pest species (Jeschke and Nauen 2008). Their intrinsic toxicity to honey bees differ with *N*-nitroguanidine neonicotinoids such as imidacloprid, thiamethoxam and clothianidin displaying a two to almost three orders of magnitude higher acute intrinsic toxicity compared to the *N*-cyanoamidine compounds thiacloprid and acetamiprid (Iwasa *et al.* 2004). As the compounds showed a similar nanomolar binding affinity to their molecular target (Nauen *et al.* 2001), the difference in toxicity has to have another origin and is part of the research addressed in this thesis.

The concerns about honey bee health and protection in the scientific, public and political area also expand to non-*Apis* species, e.g. bumblebees and solitary bees. In the EU wild bee species

are intended to be included in the risk assessment scheme for crop protection products in the future (EFSA 2013). Thus, the identification of the biochemical and molecular mechanisms underlying sensitivity towards insecticides would be beneficial for the targeted design of bee-friendly compounds and the mechanistic explanation how various bee species interact with insecticides.

This introductory chapter focuses on the biology of *A. mellifera* and briefly summarizes the biology of two other bee pollinators, *Bombus terrestris* L. and *Osmia bicornis* L. In addition, the factors affecting honey bee health and the toxicity testing scheme for crop protection products on bees in the EU are outlined. The research activities of this thesis mainly focuses on the impact of honey bee cytochrome P450 monooxygenases (P450s) in the detoxification of neonicotinoid insecticides. Hence, these topics are further introduced and a general overview of the detoxification enzyme systems is depicted. Finally, the objectives of this thesis are outlined.

1.1 Bee pollinators

Pollination is an essential step involved in the sexual reproduction of angiosperms (flowering plants) in which the male gamete pollen is transferred to the female reproductive organs thus enabling fertilization and the production of seeds (Lord and Russell 2002). While some plants are able to self-pollinate, others depend on cross-pollination mediated by wind, water or animals. A few vertebrates and a large number of insect species play a key role in the pollination of plants by transferring pollen from plant to plant (Klein *et al.* 2007, Whelan *et al.* 2009, Ollerton *et al.* 2011). These animals are called pollinators and are involved in the pollination of over 90 % of the modern angiosperms worldwide (Kearns *et al.* 1998). Insects are particularly important pollinators providing pollination as an ecosystem service to 87 out of 115 leading crops worldwide (Klein *et al.* 2007). These species include lepidopterans, flies, ants, wasps and bees. The overall value of animal pollination of agricultural crops was estimated to range between US$ 235 billion - US$ 577 billion in 2015 which reflected 5-8 % of the global crop production (IPBES report 2016). The particular value of bee pollination on crop production has been recently estimated by comparing the data from 90 studies and 1,394 crop fields on crop-visiting communities to range between US$ 3,251 ha^{-1} (s.e.=$547, range $7–14,252) for wild bees and US$ 2,913 +/- 574 ha^{-1} (range $ 0 – 18,679) for honey bees (Kleijn *et al.* 2015).

1.1.1 Bees

Bees are insects (Insecta) belonging to the order of Hymenoptera (suborder Apocrita, superfamily Apoidea) and are classified in the phylum of arthropods with 17,533 species described in 1,234 genera and subgenera worldwide (Michener 2007). However, the total number of bee species is estimated ≥ 20,000 (IPBES report 2016). In 2006, the oldest known

amber fossil of an ancient bee, *Melittosphex burmensis*, was found in Northern Myanmar (Poinar and Danforth 2006). Interestingly, this ~ 100-million-year-old excellently preserved male bee carries characteristics of both, bees and wasps. Therefore, this bee is suggested to be a remote ancestor and the evolutionary link between bees and wasps (Poinar and Danforth 2006). Previously, the stingless bee *Trigona prisca* found preserved in 75 to 92-million-year old amber in New Jersey dated the origin of bees back to the cretaceous period (Michener and Grimaldi 1988), the period when angiosperms are presumed to have evolved (for detailed information see review of Friis *et al.* 2010).

1.1.2 The western honey bee, *Apis mellifera*

Among the large diversity of bee species are honey bees (genus of *Apis*). All *Apis* species are eusocial bees and own structures to collect pollen, e.g. corbiculae (pollen baskets) and modified legs (Winston and Michener 1977). The eusocial trait of the colony is characterized by overlapping generations, cooperative brood care and a non-reproductive worker caste (Wilson and Hoelldobler 2005).

Today, the genus of *Apis* includes eleven species: *Apis mellifera*, *Apis cerana*, *Apis koschevnikovi*, *Apis nuluensis*, *Apis nigrocincta*, *Apis andreniformis*, *Apis florea*, *Apis dorsata*, *Apis laboriosa*, *Apis breviligula* and *Apis binghami* including various subspecies (Michener 2007, Crane 2009).

The most prominent and commonly managed representative of *Apis* species is the western honey bee *A. mellifera* (Linneaus, 1758) with a large number of subspecies natively distributed in Europe, Africa and the Middle East (Garnery *et al.* 1992, Crane 2009, Whitfield *et al.* 2006; Han *et al.* 2012). Today Asia and Africa are discussed as possible evolutionary origins based on morphological and molecular analyses (Garney and Solignac 1992, Whitfield *et al.* 2006, Han *et al.* 2012) with the latest evidence pointing to an Asian origin (Wallberg *et al.* 2014).

Honey bees are unique insects with a high value for mankind in terms of ecosystem services and manufactured products. The earliest evidence for the interaction of humans and bees are recorded in cave paintings which are approximately 7000 years old (Crane 1999). Back then as today humans have valued the products manufactured by bees such as honey and wax (Zumla and Lulat 1989, Crane 1999). Apiculture has been continuously developing throughout history (Crane 1999) and today beekeepers manage honey bee colonies preferentially in different hive systems that are relatively easy to handle with some training (Figure 1).

The development, life cycle and in hive dynamics of the honey bees depend on various factors such as genetics (Kraus *et al.* 2005, Tarpy *et al.* 2013), subspecies (Harbo *et al.* 1981, Nunes-Silva *et al.* 2006), in hive temperature (Fukuda and Sakagami 1968, Tautz *et al.* 2003), climate (Switanek *et al.* 2017, Flores *et al*. 2019) and nutrition (Brotschneider and Crailsheim 2010, Di Pasquale *et al.* 2013).

Figure 1 Example of a bee hive used by bee keepers (left) and inside organization of the combs in the hive (right)

Honey bees are holometabolous insects which undergo metamorphosis. They go through four distinct life stages: egg, larva, pupae and adult. Honey bees are haplodiploid meaning that fertilized eggs develop into female worker bees and unfertilized eggs into drones (Dzierzon 1845). With advancing molecular techniques, the protein encoded by the gene *complementatry sex determiner (csd)* was elucidated as the primary signal determining the sex in honey bees (Beye *et al*. 2003).

The average development time at the normal brood nest temperature of 35 °C depends on their caste. The queen is the largest individual in the colony with an average developmental time of 16 days (Table 1), whereas worker honey bees are the smallest individuals and need 21 days to complete development (Table 1). Both, the queen and worker honey bees own a sting attached to a venom sac for defensive purposes. Drones are medium-sized and have a development cycle of 24 days (Table 1) (Jay 1963, Lee and Winston 1985, Winston 1991). Worker honey bees have degraded reproductive organs and are not able to mate (Winston 1991). The tasks performed by the worker bees depend on their age; however, worker bees are flexible in changing their actual task if circumstances make it necessary (Robinson 1992, Huang and Robinson 1996). Immediately after hatching young worker bees perform cleaning tasks within the hive. Next, they serve as nurse bees for the developing brood. Afterwards, they are involved in honey processing, comb building and defend the hive entrance as guardians (Winston 1991). The last period of their life worker honey bees spend as foragers and collect pollen and nectar from plants (Roesch 1925, Lindauer 1952, Winston 1991). Foraging worker honey bees use dances (e.g. the waggle-dance) as a form of communication to help guiding their nest mates to promising flower sources (von Frisch 1946, von Frisch 1967).

Table 1 Appearance, number of individuals per colony in spring/summer and development time of the queen, worker bees and drones

			
Gender	**Queen ♀**	**Worker bee ♀**	**Drone ♂**
Weight after emerging[1,2]	178 – 292 mg	81 – 151 mg	196 – 225 mg
Individuals per colony	1	20,000 - 80,000	up to 10% of the total population
development stages			
Egg	day 1-3	day 1-3	day 1-3
Larva	day 4 - 9	day 4 - 9	day 4 - 9
Pupa	day 10 - 15	day 10 - 20	day 10 – 23
Adult[3]	day 16	day 21	day 24

([1] Jay 1963, [2] Lee and Winston 1985, [3]Winston 1991)

In spring the colony starts to raise new brood while the overwintering bees start to pass away. The average life span of a worker bee depends on the season and varies with 30 – 60 days in fall and spring, 15 - 38 days in summer and 140 and more days during the winter (reviewed by Remolina and Huges 2008), while the queen lives on average between one to two years (reviewed by Page and Peng 2001). The size of a colony can amount 20,000 - 80,000 worker bees and 300 - 3,000 drones in early summer. The sole task of drones is to mate with a young queen and they die soon after a successful mating. The queens are polyandrous and mate in only one period of their life with an average of 10 – 12 drones during one or more mating flights (Woyke 1960, Woyke 1964). The perennial colony reproduces by swarming in late spring and early summer with the old queen leaving the colony with roughly half or the worker bees while leaving the hive to a newly raised queen (Winston 1991). The worker honey bees determine which fertilized egg is supposed to develop into a future queen by feeding the larvae higher amounts of a different diet, the so called royal jelly (Winston 1991). In late summer/autumn the colony size decreases and the worker bees expel the remaining drones. The new generation of winter bees is physiologically and behaviorally different from the summer bees and have an enlarged life span. Their major task is to ensure the overwintering of the queen by forming a thermoregulating cluster around her (Doeke *et al*. 2015).

Honey bees are polylectic meaning that they forage on a large variety of flower species. The ecosystem service provided by bees has become indispensable for the pollination of various crops such as nuts, fruits and vegetables (Klein *et al.* 2007). In modern agriculture crop protection products such as insecticides, fungicides and herbicides are widely used to combat pests, unwanted diseases and weeds, respectively. Over the last years the number of publications on the effects of crop protection products, especially insecticides, on honey bees has

continuously grown. However, less is known about the interaction of bees with insecticides on the molecular and biochemical level. This topic will be further elaborated in the following chapters as it is the main research objective of this thesis.

1.1.3 The buff-tailed bumblebee, *Bombus terrestris*

Bees belonging to the Apoidea superfamily (except for the honey bee) are also referred to as wild bees. Among them are bumblebees with 250 described species worldwide (Williams *et al.* 2008). Depending on the species, bumblebees live in colonies of 20 to up to 1800 individuals with a queen (Cueva del Castillo *et al.* 2015); however, their level of sociality is lower compared to honey bees (Goulson 2003, Sadd *et al.* 2005).

One of the most intensively studied bumblebee species with a high economic importance is the buff-tailed bumblebee *Bombus terrestris* (Linneaus, 1758).

Under temperate conditions, their annual life cycle starts in February or early March when the young fertilized queens emerge from hibernation. After finding a suitable nesting site the queen rears the first offspring herself by laying a few diploid eggs that develop into female workers (Alford 1975). Worker bumblebees share labor to build and maintain the annual colony (Goulson 2003). Reproduction takes place at the end of the annual colony cycle. At a switch-point the queen biases her offspring production from laying diploid eggs developing into workers and queens towards laying haploid eggs that develop into drones (Duchateau and Velthuis, 1988; Holland *et al.* 2013). Both virgin queens and drones leave the nest after maturation for mating purposes.

The buff-tailed bumblebee has a furry black body with two yellowish brown stripes and a white tip at the abdomen (Figure 2, right picture). Workers are smaller in size compared to the large queen and female bumblebees own a sting attached to a venom sac for defensive purposes.

Figure 2 Example of a comercially available *B. terrestris* colony (left) and inside view of the colony with worker bumblebees (right)

Bumblebees are polylectic and provide important pollination services to a large variety of flowering plants (Rasmont *et al.* 2008). In 1987 the first commercial supplier of bumblebees, Biobest, was founded followed by other companies (Velthuis and van Doorn 2006). The commercial availability of colonies (Figure 2, left picture) enabled the large-scale pollination of several crops and especially improved tomato production in greenhouses (Velthuis and van Doorn 2006).

B. terrestris is commonly found throughout Europe, North Africa and on some Mediterranean and Atlantic Islands (Widmer *et al.* 1998). Due to managed pollination, *B. terrestris* has also become an invasive species in several countries and regions such as Japan (Matsumara *et al.* 2004) or South America (Schmid-Hempel *et al.* 2014).

Although *B. terrestris* is detailed studied in terms of behavioral and physiological aspects, less is known about the detoxification capacity of bumblebees to metabolize xenobiotics, e.g. insecticides. Bumblebees are intended to be included in the risk assessment scheme of crop protection products in the EU in the future (EFSA 2013). Thus, more knowledge about their biology can be helpful to support method development for regulatory purposes. Moreover, knowledge about their physiology could be valuable for the targeted design of bumblebee-friendly insecticides.

1.1.4 The red mason bee, *Osmia bicornis*

A species within the group of solitary bees that is used for ecotoxicological studies is the red mason bee, *Osmia bicornis* (Linneaus, 1758) (syn. *Osmia rufa*) (Westrich and Dathe 1997). The red mason bee is a univoltine Apoidea species belonging to the family of Megachilidae and is natively distributed throughout Europe. In contrast to eusocial bees, every female is fertile and builds a nest by herself, preferably in pre-existing tube structures (i.e. in bricks or deadwood) (Raw 1972). The female bees are monogamous and thus only mate once while males mate multiple times (Raw 1976). Afterwards, fertilized females scout for a nesting site and collect pollen and nectar that feed the developing larvae. They lay eggs in the nesting tube, each separately in build cylindrical cells on top of a pollen and nectar provision, with diploid eggs developing into females placed at the rear of the tubular cavity and haploid eggs developing into males placed more closely towards the entrance (Raw 1972, Szentgyoergyi and Woyciechowski 2013). Finally, they plug their nest with mud or similar material (Raw 1972). About one week after oviposition the larvae hatch, develop and enter the pupal stage by spinning a cocoon (Fig. 3, right picture) (Raw 1972). They overwinter as fully developed adults and emerge in early spring with males hatching first, followed by the females (Raw 1972, Szentgyoergyi and Woyciechowski 2013).

The red mason bee has a furry red body with a black tip on the abdomen. Females are larger in size than males (Figure 3) and their gender can be distinguished by their body size, antennae length and the feature that male bees have a white-colored forehead.

Figure 3 Female *O. bicornis* (left), male individual (middle) and close-up view of a cocoon (right)

A few *Osmia* species are important managed pollinators for a variety of early-flowering orchard crops (Bosch and Kemp 2002, Bilinski and Teper 2004). The fact that they can be easily reared under laboratory conditions has made *O. bicornis* a suitable candidate for various types of ecotoxicological studies. So far, less is known about the effects of crop protection products on *O. bicornis* as well as their endogenous detoxification capacity towards xenobiotics. The red mason bee is proposed to be included in the risk assessment scheme for crop protection products in the EU (EFSA 2013). Thus, further knowledge about the biology of this solitary bee is required to support the development of regulatory testing methods. Additionally, a detailed physiological understanding could promote the design of bee-friendly insecticides.

1.2 Factors affecting honey bee health

In general, there has been rising concern about an alleged decline of pollinators worldwide (Biesmeijer 2006, Goulson *et al.* 2007, Potts *et al.* 2010). As previously mentioned, honey bees have to face a number of factors potentially affecting their health. In particular pest and diseases have the potential to affect the health of honey bees (reviewed by Genersch 2010b). One of the most destructive parasites found in honey bee colonies worldwide (except Australia) is the ectoparasite *Varroa destructor*. In the past, *V. destructor* was described to feed on the hemolyph of both, adult and immature honey bees, thus weakening the individual (Rosenkranz *et al.* 2010). However, the latest reseach indicated the fat body tissue is the primary feeding site of this ectoparasite (Ramsey *et al.* 2019). Moreover, virus diseases can be transmitted by the mite, such as the deformed wing virus (DWV), adding a further pressure to the health of the colony (Genersch 2010b). If the infestation is too high the colonies become weak and will not survive.

In total, 18 different viruses could be isolated from honey bees affecting their health on various physiological levels (Chen and Siede 2007). Beside viruses, honey bees can suffer from bacterial or fungal pathogens and the related diseases often have a destructive influence on the

colony level. Among the most prominent pathogens found in honey bees are the bacterial pathogen *Paenibacillus larvae* causing American foulbrood (Genersch 2010c) or the microsporidia *Nosema ceranae* which causes nosemosis (Higes *et al.* 2006, Higes *et al.* 2007).
The global trade of bee hives and apicultural products has allowed invasive species to be spread more easily, e.g. *V. destructor*. The latest example of an invasive species strongly affecting honey bees is the small hive beetle *Aethina tumida* (Murray 1867, Lundie 1940). This devastating "globetrotter" from Africa has been introduced to other regions of the world and was first observed in the EU but successfully eradicated in Portugal in 2004 (Murilhas 2004, Valério da Silva 2014). In 2014, this pest was confirmed in Italy (Palmeri *et al.* 2014) with further records in 2015 and 2016 (Rivera-Gomis *et al.* 2017). The larvae of this pest destroy the comb structure completely by eating the pollen, honey and bee brood before leaving the hive to pupate outside in the soil. Cuthbertson *et al.* (2013) have recently reviewed the biology of the small hive beetle and control measures required to prevent spreading of the pest.
Other factors influencing honey bee colony health important to mention include agricultural and bee keeping practices, the use of crop protection products or environmental factors (e.g. climate, diet, habitat) (AFSSA 2009). In summary, honey bee health depends on various factors that influence or synergize each other to a lower or higher degree. In the worst case scenario, a honey bee colony that is affected by such factors may eventually collapse, resulting in an economical damage for apiculture and agriculture.

1.3 Crop protection products and the risk assessment on bees in the European Union

Crop protection products are important tools in modern agriculture applied to protect crops from various threats. Among them are herbicides used to combat the growth of unwanted plants or fungicides which are applied to prevent fungal diseases (Oerke 2006). The use of insecticides is beneficial to both agriculture and public health. Insecticides are applied to protect crops from pests and to increase their productivity, quality and yield, but also for vector control, e.g. to combat vector-borne diseases transmitted by mosquitos (Nauen 2007).
The discovery and development of modern crop protection products is time-intensive and it takes an average of 11.3 years and US$ 268 million investment costs for a new candidate molecule from its synthesis through the development and registration until it is first launched to the market and only one out of about 160,000 molecules pass this process successfully (Phillips McDougall 2016).
Crop protection products have to meet high standards towards human and environmental safety and are strictly regulated in the EU. Bee pollinators may be exposed to crop protection products orally or topically by residues or direct overspray of the products on the flowers by foliar application or orally by residues of systemic compounds that are translocated to nectar, pollen or guttation fluid. The risk assessment of crop protection products on bees is a part of the

regulatory requirements for the registration of each crop protection product in the EU. In addition, toxicity testing may be of academic interest to mechanistically understand how bees interact with crop protection products.

The requirements stipulated therein include the risk assessment of crop protection product for bees in the EU. Each crop protection product that is going to be introduced to the market or going through a registration renewal process ten years after the first approval (or 15 years in case of low-risk substances) after 2009 is assessed under the regulation EC No 1107/2009. Currently, the honey bee *A. mellifera* is the predominantly used species included in the toxicity testing cascade and there are well-established testing methods available. These testing methods are developed, validated and published as guidelines or guidance documents under the auspices of the Organization for Economic Co-operation and Development (OECD) or European and Mediterranean Plant Protection Organization (EPPO) and ensure reliability and reproducibility of the testing methods worldwide.

In case of honey bees, the ultimate goal is to protect the colony from adverse effects that might be owed by a product. The risk assessment of crop protection products on bees follows a tiered approach and always starts at the lower tier level (Tier 1) with the assessment of the intrinsic acute oral and contact toxicity on adult honey bees (OECD 213 and OECD 214, respectively). Figure 4 shows an example of the experimental setup for the assessment of the acute oral or contact toxicity of crop protection products on adult honey bees in the laboratory.

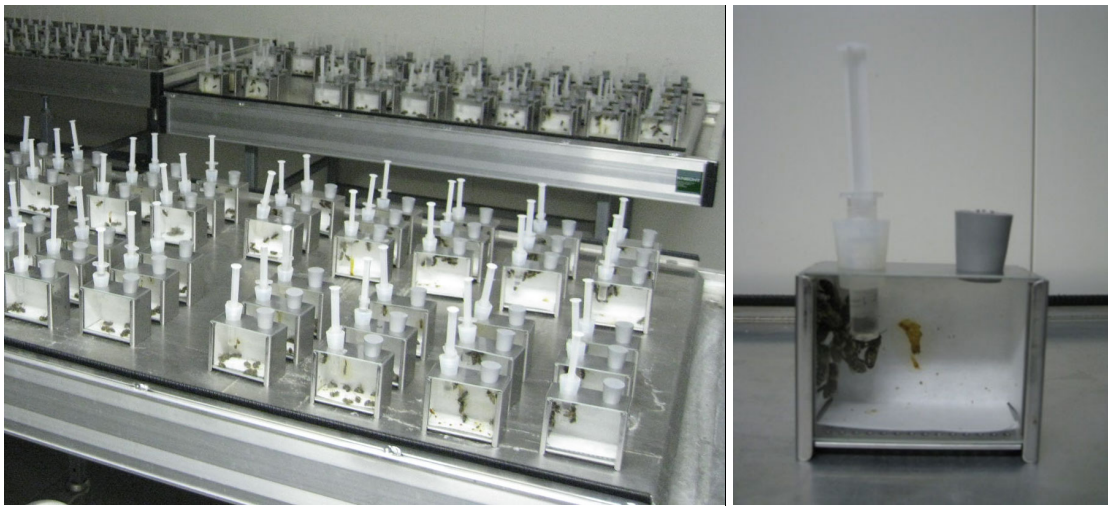

Figure 4 Setup of a honey bee acute oral or contact toxicity test (OECD 213 and 214) in a climate chamber (left) and closer view of a testing unit (right)

The acute toxicity to larvae after single exposure (OECD 237) or chronic toxicity to larvae and adults (OECD 239 and OECD 245, respectively) are assessed as part of the risk assessment. The results obtained from these laboratory tests allow an important initial evaluation of the intrinsic toxicity of a crop protection product. Very recently methods to evaluate the acute intrinsic contact (OECD 246) and oral (OECD 247) toxicity of crop protection products on bumblebees

have been published by the OECD. Testing methods to assess the chronic toxicity to bumblebees or to determine the acute contact and oral toxicity of crop protection products on *Osmia* spp. are currently under development by ring-testing groups.

The testing cascade for regulatory purposes may include higher tier testing in a semi-field setup considered as Tier 2. These tests are performed to assess the possible effects of a crop protection product on the honey bee colony in a confined, field-mimicking setup with bees actively foraging on a treated crop in a tunnel. While a test performed according OECD guidance document 75 focusses on the effects of crop protection products on honey bee brood development under semi-field conditions (OECD 75), a test performed according to OEPP/EPPO guideline 170 (OEPP/EPPO 170) aims to analyze the effects of crop protection products on the colony level in more general terms. Moreover, samples of bee relevant matrices, e.g. pollen and nectar, could be collected to determine the respective residue levels of the test item in order to prove exposure or to use the data for refinements of the risk assessment. Additionally, colony feeding studies could be performed to assess the effects of insecticides as for instance insect growth regulators on the developing bee brood (Oomen *et al.* 1992, Schmitzer and Lueckmann 2013).

Complex field trials with free-foraging bees are considered as Tier 3 in the EU and can be conducted to analyze the possible effects of crop protection products on the colony level under real field conditions. These studies are solely performed to answer very specific questions that cannot be addressed by Tier 1 and 2 testing methods.

In general, tests for regulatory purposes are normally performed according to good laboratory practice (GLP) and are often accompanied by an analytic verification of the used dosages of the test item.

In 2013, the European Food Safety Authority (EFSA) published a draft guidance document on the risk assessment of crop protection products on bees including *Bombus* spp. and solitary bees as well as honey bees (EFSA 2013). This guidance document is not yet officially implemented as it includes testing methods and requirements that are impractical and unworkable due to the non-availability of the required testing methods or requirements for higher tier testing which are technically impossible to fulfill (ECPA 2017). The full implementation would have the consequence that most of the crop protection products would not pass the risk assessment as the proposal is based on overly conservative assumptions that are linked to unrealistic protection goals (ECPA 2017).

1.4. Insecticides targeting the nicotinic acetylcholine receptor (nAChR) as agonists and their impact on honey bees

Insecticides can target the nervous system and muscles, growth and development, respiration, midgut or other non-specific sites within the insect. They can also affect either the developing

larvae or the adult insects. The Insecticide Resistance Action Committee (IRAC) classified all commercially available products according to their mode of action into groups (Sparks and Nauen 2015).

A prominent neuronal target involved in the fast excitatory synaptic signal transmission is the nicotinic acetylcholine receptor (nAChR). nAChRs are membrane-integrated pentameric receptors which belong to the superfamily of cys-loop ligand gated ion channels (Figure 5, right picture) and are found in vertebrates and invertebrates (Tomizawa and Casida 2001, Jeschke *et al.* 2013). The extracellular binding of the endogenous agonist acetylcholine (ACh) causes a conformational change in the receptor resulting in its opening and the influx of cations, in particular sodium ions, causing the depolarization of the postsynaptic plasma membrane (Tomizawa and Casida 2003) (Figure 5, left picture).

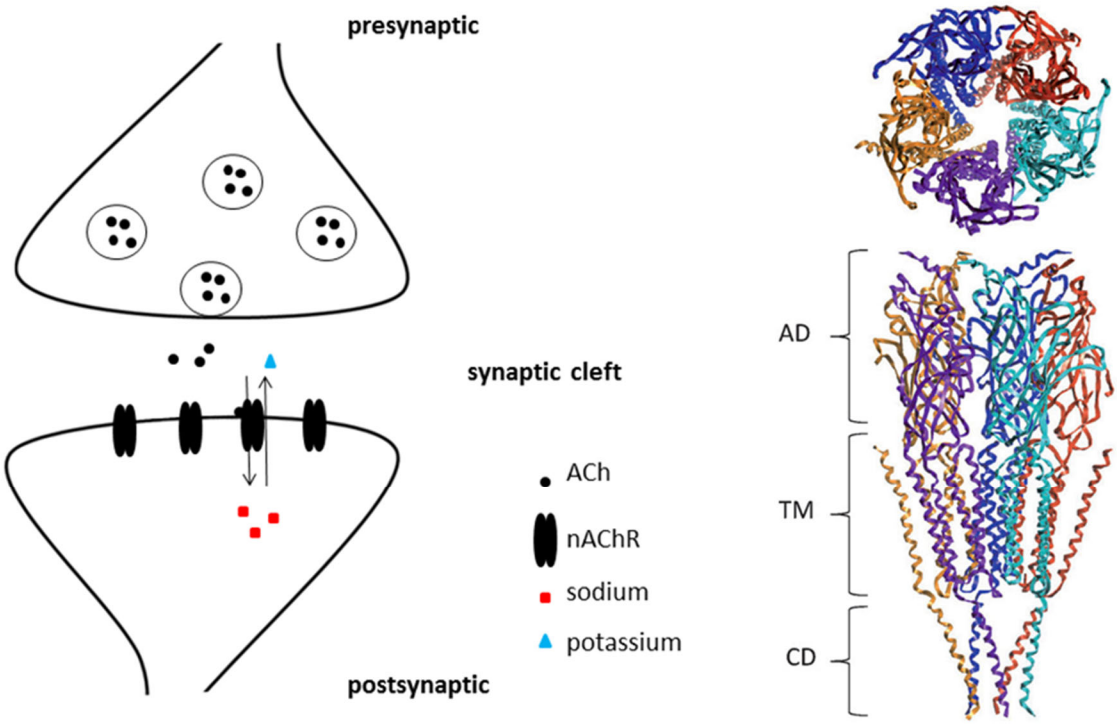

Figure 5 Simplified illustration of the sodium influx and potassium eflux after binding of the endogenous neurotransmitte ACh to postsynaptic nAChRs (left) and 3D structure of *Torpedo marmorata* nAChR in a side view (top) and along the chanel including the antagonist binding domain (AD), transmembrane domaine (TD) and cytoplamic domain (CD) (right picture, taken from Jeschke *et al.* 2013)

The number of receptor subunits varies among species. Recently 11 genes encoding nAChR subunits have been identified in the honey bee genome (Jones *et al.* 2006). Insecticides targeting the nAChR in the central nervous system of insects as competitive modulators are listed in IRAC class 4. Among them are five subgroups including neonicotinoids, nicotine, sulfoximines, butenolides and mesoionics.

1.4.1 Neonicotinoids

Neonicotinoid insecticides target the postsynaptic nAChRs of insect's as partial agonists and are classified in IRAC subgroup 4a, the neonicotinoids (Jeschke and Nauen 2008, Sparks and Nauen 2015). In the early 1970s, Shell discovered nitromethylen heterocycles acting on the nAChR leading to synthesis of nithiazine which is considered the lead structure of the neonicotinoids today (Jeschke and Nauen 2008, Jeschke *et al.* 2013). Imidacloprid was the first commercially available representative of this novel class of synthetic insecticides launched to the market in 1991 (Jeschke *et al.* 2008). Currently, seven compounds are commercially available (imidacloprid, thiamethoxam, clothianidin, dinotefuran, nitenpyram, thiacloprid and acetamiprid) for the control of a broad spectrum of sucking and certain chewing insects in a wide range of applications (Jeschke and Nauen 2008). Neonicotinoids mimic the endogenous ligand ACh. They reversibly bind to postsynaptic nAChRs located in the insects` central nervous system and thus initiate signal transmission which results in a continuous influx of sodium ions causing paralysis and death of the insect (Jeschke *et al.* 2013).

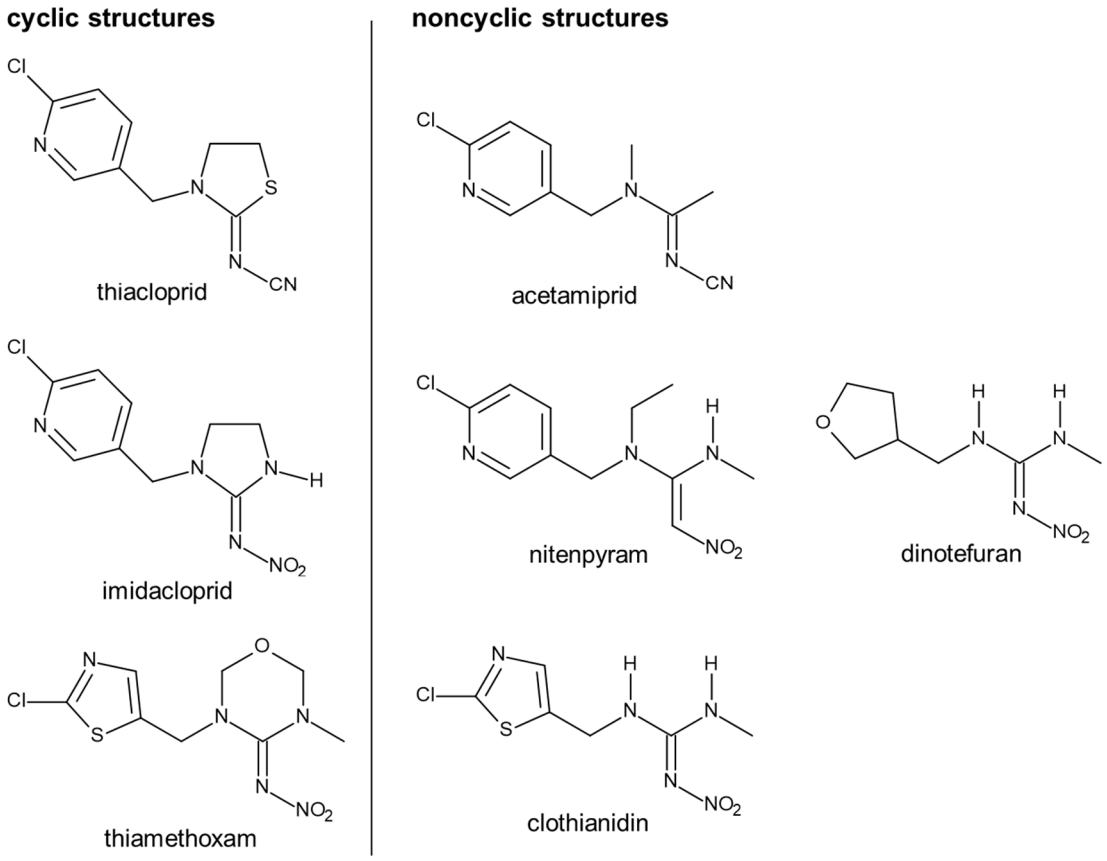

Figure 6 Structure of the the seven comercially available neonicotinoid insecticides categorized into compounds with ring systems and non-cyclic structures

The name 'neonicotinoids' was proposed to distinguish between the naturally occurring (S)-nicotine and this novel class of synthetic compounds as the molecules shared the same mode of

action (Tomizawa and Yamamoto 1993). Due to their high efficiency against target species and low intrinsic toxicity to vertebrates, neonicotinoids quickly became a very successful class of chemistry reflected by a market share of 24 % in 2007 (Jeschke *et al.* 2011). Moreover, they own systemic properties meaning that the compound can be taken up by the plant and is distributed within the plant tissue. Neonicotinoids can be distinguished by their structure and pharmacophore system. Whilst imidacloprid, thiacloprid and thiamethoxam have a cyclic structure; acetamiprid, dinotefuran, clothianidin and nitenpyram display an open-chain structure (Figure 6). Clothianidin is a metabolite of thiamethoxam metabolism in insects and plants (Nauen *et al.* 2003).

The different pharmacophore systems of the neonicotinoids make a difference when it comes to their intrinsic toxicity on honey bees, although the compounds share a similar binding affinity to their molecular target (Nauen *et al.* 2001). The *N*-nitro-substituted neonicotinoids imidacloprid, thiamethoxam, clothianidin and dinotefuran display a two to three orders of magnitude higher intrinsic toxicity to honey bees compared to the *N*-cyano-substituted molecules thiacloprid and acetamiprid (contact LD_{50} values (24 h) listed in table 2) (Iwasa *et al.* 2004). In case of imidacloprid it was demonstrated that the olefin and di-hydroxy metabolite display a comparable intrinsic toxicity as the parent compound itself (Nauen *et al.* 2001).

Table 2 IUPAC names, pharmacophore sytem and contact toxicity of neonicotinoids on *Apis mellifera*

Compound	**IUPAC name**	**Contact LD_{50} (24 h) µg a.i./ honey bee** (taken from (Iwasa *et al*., 2004)	**Pharmacophore system**
Thiacloprid	[3-[(6-chloropyridin-3-yl)methyl]-1,3-thiazolidin-2-ylidene]cyanamide	14.6	cyano
Acetamiprid	N-[(6-chloropyridin-3-yl)methyl]-*N*'-cyano-*N*-methylethanimidamide	7.07	cyano
Thiamethoxam	(*NE*)-*N*-[3-[(2-chloro-1,3-thiazol-5-yl)methyl]-5-methyl-1,3,5-oxadiazinan-4ylidene]nitramide	0.0299	nitro
Clothianidin	1-[(2-chloro-1,3-thiazol-5-yl)methyl]-3-methyl-2-nitroguanidine	0.0218	nitro
Imidacloprid	*N*-[1-[(6-chloropyridin-3-yl)methyl]-4,5-dihydroimidazol-2-yl]nitramide	0.0179	nitro
Dinotefuran	2-methyl-1-nitro-3-(oxolan-3ylmethyl)guanidine	0.0750	nitro
Nitenpyram	(*E*)-1-*N*'-[(6-chloropyridin-3-yl)methyl]-1-*N*'-ethyl-1-*N*-methyl-2-nitroethene-1,1-diamine	0.138	nitro

Synergist bioassays and metabolic fate experiments clearly indicated that cytochrome P450 monooxygenases play an important role in the oxidative metabolism of neonicotinoids (Iwasa *et al.* 2004, Suchail *et al.* 2004a, Suchail *et al.* 2004b, Brunet *et al.* 2005). Moreover, certain ergosterol biosynthesis inhibiting fungicides (EBI) have been identified as potent synergists that significantly increase the toxicity of *N*-cyano-substituted neonicotinoids by inhibiting enzyme function (Iwasa *et al.* 2004). So far, the key enzymes involved in neonicotinoid metabolism in bee pollinators remained elusive and their role in the oxidative metabolism of selected neonicotinoids will be further outlined in chapter 3.

The possible adverse effects of neonicotinoids on honey bees and other bee species such as the bumble bee and red mason bee have been addressed by upcoming publications on an almost weekly basis and are assumed by some to be involved in compromised health of honey bees and decline of wild bee pollinators (Whitehorn *et al.* 2012, Di Prisco *et al.* 2013, Feltham *et al.* 2014, Sandrock *et al*. 2014). Due to rising concerns of the effects of neonicotinoids with a high intrinsic toxicity to bees, an independent risk assessment based on recent product submissions and peer-reviewed publications was conducted by EFSA. As the risk of imidacloprid (EFSA conclusion 2013a), thiamethoxam (EFSA conclusion 2013b) and clothianidin (EFSA conclusion 2013c) to bees was indicated or could not be excluded, the EU restricted certain uses of products containing these compounds on bee-attractive crops from 1st December 2013 (EU regulation No. 485/2013). Upon request by the European Commission, an updated risk assessment was conducted by EFSA taking into consideration new studies, monitoring activities and research on honey bees, bumblebees and wild bee species (EFSA conclusion 2018a, EFSA conclusion 2018b, EFSA conclusion 2018c). EFSA concluded that some uses pose a risk to bees and as a result, the Standing Committee of the European Commission decided on a complete ban of field-use of imidacloprid, clothianidin and thiamethoxam on the 27th of April 2018.

The fact that a molecule displays a high intrinsic toxicity to bees in laboratory bioassays is not necessary reflected in its field use. A recently published large-scale field study examining the effects and residues of clothianidin used as an oilseed rape seed coating showed no adverse effects on honey bees, bumble bees and red mason bees under field conditions, respectively (Rolke *et al.* 2016, Sterk *et al.* 2016, Peters *et al.* 2016). As the compounds have been thoroughly tested by the ecotoxicological departments of the respective owner companies, they can be considered bee-safe when the products are used in compliance with the respective product label instructions.

1.4.2 Nicotine

Nicotine (Figure 7) is a secondary plant metabolite found in plants belonging to the nightshade family and is well known for its use as medicine, a legal drug and as an insecticide (Ujvary 1999, Yamamoto 1999). This alkaloid selectively acts on the nAChR in the insects` nervous system and is assigned to IRAC subgroup 4B, nicotine (Sparks and Nauen 2015).

A study conducted by Singaravelan *et al.* (2006) demonstrated that naturally occurring concentrations of nicotine in plants do not affect honey bee colony fitness and reproduction. In conclusion, honey bees must have evolved metabolic pathways to detoxify this alkaloid.

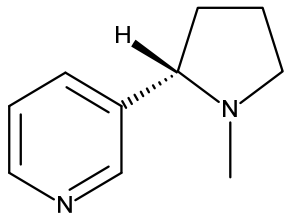

Figure 7 Structure of nicotine

A metabolic and proteomic profiling study confirmed the participation of phase I detoxification enzymes, especially cytochrome P450 monooxygenases, in nicotine metabolism resulting in the dietary tolerance of nicotine in worker honey bees (du Rand *et al.* 2015). In addition, a pharmacokinetic study tracking [^{14}C]-labelled nicotine demonstrated the rapid metabolism of nicotine by honey bees (du Rand *et al.* 2017) with 2`oxidation identified as the major pathway (du Rand *et al.* 2017). However, the individual honey bee P450s facilitating oxidative metabolism remain so far unknown.

1.4.3 Sulfoximines

Sulfoxaflor (Figure 8) is a systemic insecticide with a broad spectrum on sap-feeding insects that targets the insects` nAChR as an agonist (Babcock *et al.* 2011, Sparks *et al.* 2013). Containing the unique sulfoximine moiety and a few other characteristics, sulfoxaflor is structurally distinct from other insecticides binding to the same target-site (Sparks *et al.* 2013) such as the neonicotinoids and butenolides and is therefore categorized in IRAC subgroup 4C as the so far only representative (Sparks and Nauen 2015).

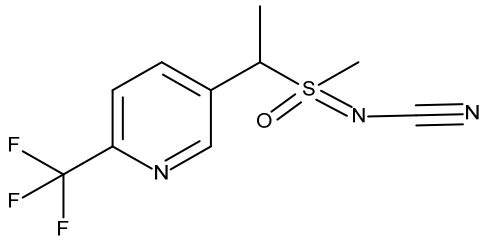

Figure 8 Structure of Sulfoxaflor

Sulfoxaflor has a high intrinsic toxicity on honey bees reflected by an acute contact and oral LD_{50} (48h) of 0.379 µg a.i./honey bee and 0.146 µg a.i./honey bee, respectively (Sparks *et al.*, 2012). Hence, the use in bee-attractive crops has to be strictly performed according to label instructions to avoid unwanted exposure to bees.

1.4.4 Butenolides

The butenolide insecticides are one of the latest invented chemical classes and categorized in IRAC subgroup 4D (Sparks and Nauen 2015) with flupyradifurone (Figure 9) as the first representative launched under the trade name SIVANTO® (Nauen *et al.* 2015, Jeschke *et al.* 2015). This systemic insecticide reversibly binds to the nAChR as an agonist and efficiently targets a broad number of sucking pests while displaying a favorable ecotoxicological profile. The naturally occurring alkaloids from the Asian medical plant *Stemona japonica* served as the lead for the development of flupyradifurone as these secondary plant metabolites display insecticidal activity (Nauen *et al.* 2015, Jeschke *et al.* 2015). A novelty of flupyradifurone is the unique butenolide pharmacophore that makes this compound distinct from other IRAC class 4 insecticides, such as neonicotinoids (Nauen *et al.* 2015, Jeschke *et al.* 2015).

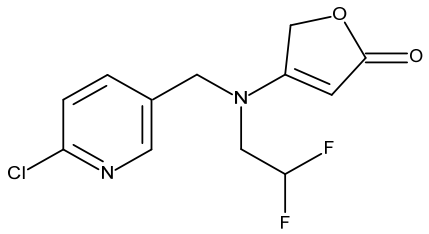

Figure 9 Structure of flupyradifurone

Flupyradifurone shows practically no toxicity to adult honey bees and bumblebees upon contact exposure (LD_{50} (48h) > 100 µg a.i./honey bee) (Nauen *et al.* 2015). Although the acute oral toxicity to adult honey bees is about one order of magnitude higher (LD_{50} (48h) 1.2 µg a.i./honey bee) (Nauen *et al.* 2015), a number of studies conducted under semi-field and field conditions showed that the use of SIVANTO® during full-flowering possess no adverse effects on honey bee colonies when the product is applied in accordance with the label instructions (Nauen *et al.* 2015, Campbell *et al.* 2016).

1.4.5 Mesoionics

Mesoionic insecticides have been recently developed for the control of hemipteran and lepidopteran pest species (Holyoke *et al.* 2015, Holyoke *et al.* 2017) with triflumezopyrim as the first representative categorized in the novel IRAC subgroup 4E, the mesoionics.

Triflumezopyrim (Figure 10) binds to the orthosteric site of the nAChR and inhibits this target in hemipteran pest species, e.g. the brown plant hopper, a major rice pest in Asia (Holyoke *et al.* 2015, Holyoke *et al.* 2017). Triflumezopyrim shows a high intrinsic toxic to honey bees with an LD_{50} value (72 h) of 0.51 µg a.i./honey bee and 0.39 µg a.i./honey bee after oral and contact exposure, respectively (Holyoke *et al.* 2015).

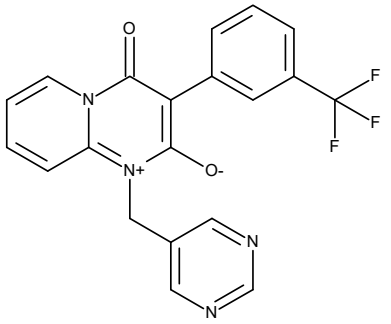

Figure 10 Structure of triflumezopyrim

Therefore, the use has to be strictly performed in compliance with the label instructions to prevent unwanted exposure to bees collecting rice pollen that may contain residues of this compound.

1.5 Resistance mechanisms of insects to insecticides

The extensive use of insecticides can lead to the development of resistance of pests by natural selection. According to the Insecticide Resistance Action Committee (IRAC), resistance is defined as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species" (McCaffery and Nauen 2006).

In the ideal situation, insecticides with different modes of action should be rotated to prevent the spread of resistance. However, in reality the situation is not that simple due to the presence of already resistant pest populations in the field and also due to a limitation in the number of products targeting the pest species while covering different modes of action.

In general, there are four principle mechanisms involved in insecticide resistance: metabolic resistance, target-site resistance, penetration resistance and behavioral resistance. Multiple and cross-resistance has been found to be caused by a number of these mechanisms acting simultaneously.

Metabolic resistance is one of the most common resistance mechanisms (Scott 1999). In this case, the pest species becomes capable of breaking down the insecticide due to the overexpression of detoxification enzymes such as cytochrome P450 monooxygenases, esterases and glutathione S-transferases. In many cases metabolic resistance is mediated by P450s (Bergé *et al.* 1998) e.g. neonicotinoid resistance in *Musca domestica* (Markussen and Kristensen 2010) and *Bemisia tabaci* (Karunker *et al.* 2008) or pyrethroid resistance in *Meligethes aeneus* (Zimmer and Nauen 2011).

Target-site resistance is characterized as the alteration of a target protein leading to physical changes in the protein that prevent the binding of the insecticides (i.e. by single or multiple amino acid substitutions). This resistance mechanism has been described for different chemical

classes including pyrethroids (Williamson *et al.* 1996, Zimmer *et al.* 2014a), organophosphates (Russell *et al.* 2004), neonicotinoids (Bass *et al.* 2011), diamides (Troczka *et al.* 2012) and benzylphenylureas (Douris *et al.* 2016). One of the most prominent target-site resistance mechanisms is knockdown resistance (*kdr*) conferring resistance to pyrethrins, pyrethroids and DDT in many pest species and vectors of human diseases (Rinkevich *et al.* 2013).

Penetration resistance can develop in pest populations causing a slower penetration of a compound through the insects` cuticle compared to susceptible insects but are generally considered of lesser importance (Ahmad and McCafferey 1999).

Behavioral resistance is characterized by the partial or complete avoidance of compounds by pest species and thus they are not or only partially exposed to the respective compound (Sparks *et al.* 1989, Wang *et al.* 2004).

So far, no insecticide resistance based on selection pressure has been described for honey bees. However, honey bees must have evolved mechanisms to overcome toxicity of certain xenobiotics.

1.6 Detoxification enzyme systems

After the exposure to xenobiotics (e.g. insecticides or phytochemicals) pharmacokinetic parameters such as absorption, distribution, metabolism and excretion (ADME) contribute to the fate of a compound within the organism and are influenced by the physiochemical properties of the compound. In the case of insects, the absorption can take place via two routes. First, compounds could penetrate through the complex cuticle that serves as a barrier between the insect and the environment. Second, the compound could be ingested orally and is then distributed with the circulating hemolymph after passing the gut wall (Smagghe and Tirry 2001).

The major site of detoxification in insects is the midgut (Smagghe and Tirry 2001); however, detoxification can also take place in other organs such as the Malpighian tubules (Figure 11) which are the insects' functional equivalent to human kidneys (Nocelli *et al.* 2016).

In general, the detoxification of a large number of endogenous and exogenous compounds is mediated by three different detoxification enzyme systems. Among them are cytochrome P450s monooxygenases (P450) and carboxylesterases (CCE) operating in phase I metabolism and glutathione S-transferases (GST) acting in phase II metabolism.

The *A. mellifera* genome encodes 10 GSTs, 24 CCEs and 46 P450s (Honey Bee Sequencing Consortium 2006, Claudianos *et al.* 2006). Although the total number of detoxification enzymes in honey bees is lower compared to other insect species such as *Drosphila melanogaster* (38 GSTs, 85 P450s and 35 CCE) or *Anopheles gambiae* (31 GSTs, 106 P450s and 51 CCE) (Claudianos *et al.* 2006), a comparative analysis of available honey bee toxicity studies

demonstrated that honey bees are not more sensitive to insecticides than other insects (Hardstone and Scott 2010).

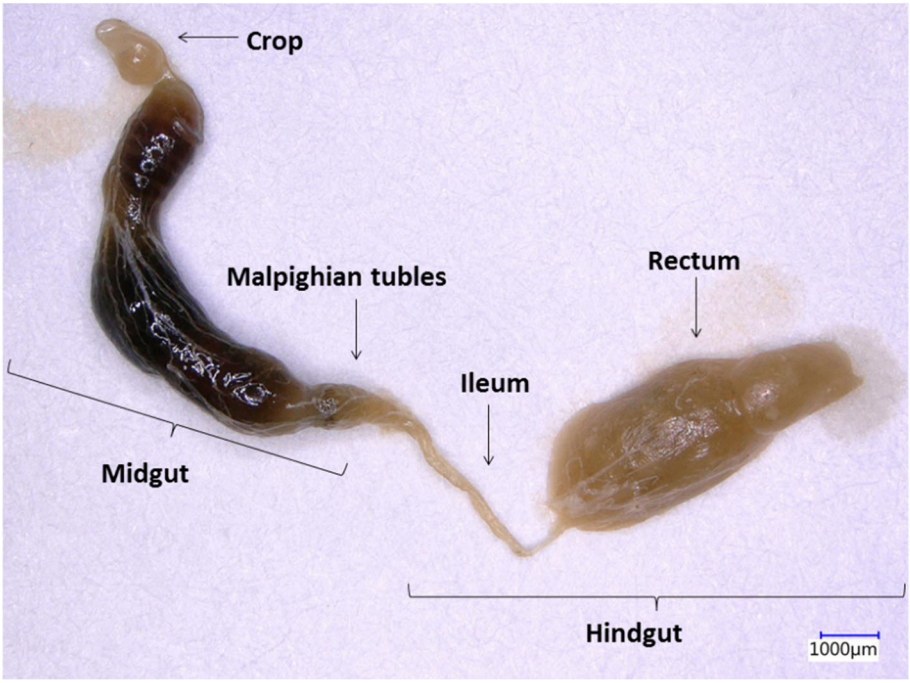

Figure 11 Preparation of the honey bee digestive system

1.6.1 Carboxylesterases

Carboxylesterases (CCE) are hydrolases that are involved in Phase I biotransformation processes. These enzymes hydrolyze molecules with an ester bond into an acid and alcohol (Wheelock *et al.* 2005). In insects, CCEs were often identified as key enzymes mediating resistance to chemical classes containing ester bonds such as organophosphates, pyrethroids and carbamates (Wheelock *et al.* 2005).

1.6.2 Cytochrome P450 monooxygenases

Cytochrome P450 monooxygenases (P450) are a superfamily of hemoproteins found in both, eukaryotic and prokaryotic organisms (Werck-Reichhart and Feyereisen 2000).

In insects, P450s and are well known to facilitate oxidative metabolism of a large number of endogenous and exogenous compounds including insecticides, but also to catalyze other reactions such as the synthesis of hormones (Feyereisen 1999). Depending on the type, insect P450s are categorized into four different clades: CYP2, CYP3, CYP4 and the mitochondrial clade (Feyereisen 2006). Especially insect P450s belonging to clade 3 were frequently identified as key enzymes conferring metabolic resistance in various insect species to different chemical classes such as pyrethroids (Zimmer *et al.* 2014b) or neonicotinoids (Karunker *et al.* 2008).

Today not much is known about the particular detoxification capacity of honey bee P450s. One reason is the problem associated with the isolation of functional microsomal fractions from honey bees. Microsomes are subcellular fractions derived from the membranes of the ER and are isolated by tissue homogenization followed by high speed sedimentation at 100,000 *g* (Claude 1969, Feyereisen *et al.* 1985). Microsomes offer a powerful tool to study P450-driven metabolism *in vitro* as they contain all endogenous membrane-bound P450s of the organism of interest. Although the preparation of active microsomal membranes from many insect species is straightforward, their isolation from the honey bee has remained challenging for over four decades. In 1974, Gilbert and Wilkinson published first work on this topic. They were not able to isolate functional microsomes from different honey bee tissues (Gilbert and Wilkinson1974) and assumed that a macromolecule located in the soluble fraction of the midgut might be responsible for the inhibition of microsomal activity upon tissue homogenization (Gilbert and Wilkinson 1975). However, the mechanism behind the inhibition could not be further clarified. In chapter 2, the problems associated with the isolation of functional honey bee microsomes are biochemically approached.

Another option to study P450-driven metabolism *in vitro* is offered by functionally expressed enzymes. In honey bees, the wider enzymatic properties of individual P450s to detoxify insecticides remain largely unknown. So far, only members of the CYP9Q-subfamily have been shown to metabolize the in-hive used miticides coumaphos and *tau*-fluvalinate (Mao *et al.* 2011). Thus, expanding the knowledge about the detoxification capacity of honey bee P450s is required to understand how they interact with xenobiotics on the biochemical and molecular level in order to promote the design of intrinsically bee-friendly insecticides. The role of individual honey bee P450s in the metabolism of certain neonicotinoid insecticides will be further addressed in chapter 3.

Additionally, clade 3 P450s were described to be involved in the metabolism of the secondary plant metabolite quercetin (Mao *et al.* 2009, Mao *et al.* 2011) or upregulated after exposure to nectar constituents (Mao *et al.* 2013). Moreover, there is compelling evidence that P450s play a crucial role in the detoxification of nicotine (Du Rand *et al.* 2015, Du Rand *et al.* 2017). Secondary plant metabolites comprise a large group of phytochemicals with more than 200,000 identified molecules (Wink 2016). They are produced by angiosperm plants for defensive purposes, to attract pollinators or seed dispersal purposes (Wink 2016, Wink 2018). Thus, molecular and biochemical studies could provide novel insights in plant-pollinator interactions.

1.6.3 Glutathione S-transferases

Glutathione S-transferases (GSTs) are an important enzyme system operating in Phase II metabolism. These enzymes are particularly known to catalyze conjugation reactions of the reduced form of glutathione (GSH) to electrophilic substrates, e.g. insecticides (Enayati *et al.*

2005). The conjugation reaction leads to an increased water solubility of the compound (Enayati *et al.* 2005) and promotes the removal of conjugates out of the cell by ABC-transporters operating in Phase III metabolism (Ishikawa 1992). It was demonstrated that GSTs are involved in the resistance of insects toward insecticides belonging to various chemical classes, e.g. pyrethroids and organophosphates (Pavlidi *et al.* 2018).

1.7 Objectives

This thesis was conducted as part of the "Bee Toxicogenomics Project" - a collaborative project between Bayer AG Crop Science Division, Rothamsted Research and the University of Exeter aiming to understand the molecular and biochemical mechanisms of sensitivity of certain insecticides towards bee pollinators with the ultimate goal to support the design of bee-friendly insecticides.

Numerous studies have been published demonstrating that P450s play a major role in the oxidative metabolism of insecticides in insect species other than bees. However, the particular detoxification enzyme systems involved in xenobiotic metabolism of honey bees remain largely unknown, which is reflected in the low number of publications on this topic.
In this thesis, the contribution of P450s to the metabolism of neonicotinoid insecticides targeting the nAChR in the western honey bee *A. mellifera* was assessed.

The successful isolation of functional microsomes from honey bees to study P450-driven metabolism *in vitro* has remained challenging over the last four decades. Chapter 2 aimed to identify the factor(s) responsible for the inactivation of P450s in honey bee microsomes. A method for the successful isolation of functional honey bee microsomes from worker bee abdomens is described and the isolated P450s are biochemically characterized.

The aim of chapter 3 was to unravel the molecular determinants of bee sensitivity to neonicotinoid insecticides in the honey bee (*A. mellifera*) and the buff-tailed bumblebee (*B. terrestris*).

The objective of chapter 4 was to establish a method to track the metabolic fate of [^{14}C]-labelled neonicotinoid insecticides *in vivo* and thus contributing to the analysis of the pharmacokinetics and metabolism of thiacloprid, acetamiprid and imidacloprid in honey bees upon contact exposure.

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Chapter 2

New approaches to old problems: Removal of phospholipase A_2 results in highly active microsomal membranes from the honey bee, *Apis mellifera*

Authors: Marion Zaworra and Ralf Nauen

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New approaches to old problems: Removal of phospholipase A_2 results in highly active microsomal membranes from the honey bee, *Apis mellifera*

Marion Zaworra[a,b], Ralf Nauen[a,*]

[a] Bayer AG, Crop Science Division, R&D, Alfred Nobel Str. 50, D-40789 Monheim, Germany
[b] University of Bonn, INRES, Molecular Phytomedicine, Karlrobert-Kreiten-Str. 13, D-53115 Bonn, Germany

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ABSTRACT

Over the last 50 years numerous studies were published by insect toxicologists using native microsomal membrane preparations in order to investigate *in vitro* cytochrome P450-(P450) mediated oxidative metabolism of xenobiotics, including insecticides. Whereas the preparation of active microsomal membranes from many pest insect species is straightforward, their isolation from honey bees, *Apis mellifera* (Hymenoptera: Apidae) remained difficult, if not impossible, due to the presence of a yet unidentified endogenous inhibitory factor released during abdominal gut membrane isolation. Thus hampering *in vitro* toxicological studies on microsomal oxidative phase 1 metabolism of xenobiotics, including compounds of ecotoxicological concern. The use of microsomal membranes rather than individually expressed P450s offers advantages and allows to develop a better understanding of phase 1 driven metabolic fate of foreign compounds. Here we biochemically investigated the problems associated with the isolation of active honey bee microsomes and developed a method resulting in highly active native microsomal preparations from adult female worker abdomens. This was achieved by removal of the abdominal venom gland sting complex prior to microsomal membrane preparation. Molecular sieve chromatography of the venom sac content leads to the identification of phospholipase A_2 as the enzyme responsible for the immediate inhibition of cytochrome P450 activity in microsomal preparations. The substrate specificity of functional honey bee microsomes was investigated with different fluorogenic substrates, and revealed a strong preference for coumarin over resorufin derivatives. Furthermore we were able to demonstrate the metabolism of insecticides by honey bee microsomes using an approach coupled to LC-MS/MS analysis of hydroxylated metabolites. Our work provides access to a new and simple *in vitro* tool to study honey bee phase 1 metabolism of xenobiotics utilising the entire range of microsomal cytochrome P450s.

1. Introduction

The metabolism and fate of xenobiotics is to a great extent driven by their detoxification and/or activation by cytochrome P450 monooxygenases (P450s), especially present in microsomal membranes (Feyereisen, 1999; Li et al., 2007; Liu et al., 2015). P450s constitute a large gene superfamily of hemeproteins present in all kingdoms of life. Microsomal P450s are membrane bound enzymes and known to catalyze a broad range of reactions in conjunction with cytochrome P450-reductase and NADPH as an electron donor (Guengerich, 2001). In insects they are for example involved in the biosynthesis of endogenous compounds such as hormones and in the detoxification of xenobiotics including pesticides (Li et al., 2007; Feyereisen, 2012). Insect and vertebrate microsomal preparations have been used for more than six decades to study the oxidative *in vitro* metabolism of xenobiotics, including insecticides (Brodie et al., 1955; O'Brien, 1959; Agosin et al., 1961; Arias and Terriere, 1962; Hodgson and Plapp Jr., 1970). Hence, standardized protocols for the isolation of functional insect microsomal fractions are available and have been straightforward (Feyereisen et al., 1985; Lee and Scott, 1989; Scott, 1996).

However, the isolation of functional microsomes from the western honey bee, *Apis mellifera*, was much more challenging and early work in 1974 demonstrated that microsomes prepared from homogenized worker bee gut preparations were not functional, whereas intact midguts expressed epoxidase, hydroxylase and *O*-demethylase activity (Gilbert and Wilkinson, 1974). Further work revealed that the lack of microsomal activity in honey bee gut preparations is due to the presence of a soluble nucleoprotein, strongly inhibiting insect microsomal membrane activity (Gilbert and Wilkinson, 1975). However, the nature of the inhibitory protein remained elusive, and for *in vitro* detoxification

* Corresponding author at: Bayer AG, CropScience Division, R&D, Pest Control, Building 6220, Alfred Nobel Str. 50, D-40789 Monheim, Germany.
E-mail address: ralf.nauen@bayer.com (R. Nauen).

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studies either intact honey bee midguts (Yu et al., 1984; Smirle and Winston, 1987; Smirle, 1993), or microsomal membranes prepared from dissected midguts and/or fat body tissue, of both adults and larvae, were used (Vidau et al., 2011; Fine and Mullin, 2017). Studies carried out more recently still referred to the presence of an inhibitory factor, and used floating honey bee abdomens - instead of dissected midguts - to investigate the interaction of piperonyl butoxide with the whole set of microsomal monooxygenases, particularly to predict potential non-target effects of insecticide/synergist combinations (Alptekin et al., 2015; Todeschini et al., 2017).

The assembly and annotation of the honey bee genome in 2006 revealed the presence of 46 P450 genes (The Honeybee Genome Sequencing Consortium, 2006), more than half of them belonging to clade 3, *i.e.* P450 subfamilies CYP6 and CYP9 (Claudianos et al., 2006). These P450 subfamilies have often been shown to be overexpressed and conferring metabolic insecticide resistance in various pest insects (Feyereisen, 2012). Despite the fact that the honey bee CYPome has been shown to be smaller than those of other insects (Claudianos et al., 2006; Feyereisen, 2011; Berenbaum and Johnson, 2015), honey bees are principally not more sensitive than other insects to insecticides across chemical classes as a recent meta analyses of literature data revealed (Hardstone and Scott, 2010). Thus suggesting metabolic capacities to detoxify a number of insecticide chemotypes (Johnson, 2015; Gong and Diao, 2017). The oxidative capacity to detoxify insecticides of different mode of action classes is further supported by *in vivo* data, showing a strong synergism of insecticide efficacy in honey bees with P450 inhibitors such as azole fungicides and piperonyl butoxide (Iwasa et al., 2004; Johnson et al., 2006, 2009).

Recent progress in molecular techniques overcomes to some extent the issue to prepare active honey bee gut microsomal membranes for toxicological studies. Cell-based functional expression of individual honey bee P450 enzymes, particularly those of subfamilies CYP6 and CYP9, allowed to investigate their individual role in the metabolism of plant secondary metabolites and compounds of ecotoxicological concern (Mao et al., 2009; Mao et al., 2011; Manjon et al., 2018). A recent study on neonicotinoid selectivity in honey bees, based on a P450 expression library encompassing all clade 3 P450s, identified a single P450, CYP9Q3 that metabolizes thiacloprid and acetamiprid, but not imidacloprid, thus helping to shed light on the bee safety of *N*-cyano substituted neonicotinoids (Manjon et al., 2018). Furthermore orthologous genes in *Bombus terrestris* and *Osmia bicornis* were shown to act in a similar way (Manjon et al., 2018; Beadle et al., 2019). Members of the CYP9Q-subfamily seem to target a broad range of insecticide chemotypes, because recently the metabolism of the in-hive used miticides *tau*-fluvalinate and coumaphos has already been demonstrated (Mao et al., 2011).

However, by studying individual bee P450s it remains unknown, if certain P450s act in concert and would possibly boost the detoxification power. Therefore we revisited the old problem originally addressed by Gilbert and Wilkinson (1974, 1975) and investigated how to make honey bee microsomes work, because the inhibiting factor still remains elusive. Establishing a method for the isolation of functional honey bee microsomes is beneficial, *e.g.* to address questions related to the detoxification of compounds of ecotoxicological concern in an *in vitro* system, thus allowing a high throughput of samples in biochemical assays. During the course of our studies we discovered that, after removal of the venom gland sting complex, isolated honey bee microsomes are highly active when incubated with both, artificial model substrates and thiacloprid.

2. Material & methods

2.1. Chemicals

All chemicals and solvents used in the study were of analytical grade. The artificial P450 model substrates 7-benzyloxy-4-trifluoromethyl coumarin (BFC), 7-methoxy-4-trifluoromethyl coumarin (MFC), 7-ethoxy-4-trifluoromethyl coumarin (EFC), 7-benzyloxymethoxy-4-trifluoromethyl coumarin (BOMFC), 7-ethoxy coumarin (EC), 7-methoxy coumarin (MC), 7-ethoxyresorufin (ER), 7-benzyloxyresorufin (BOR), 7-methoxyresorufin (MR), 7-n-pentoxyresorufin (PR), NADPH, L-glutathione (oxidized), glutathione reductase from baker's yeast (*Saccharomyces cerevisiae*), *A. mellifera* phospholipase A_2 and Bradford reagents including the protein standard bovine serum albumin were purchased from Sigma Aldrich (St. Louis, MO); 7-benzyloxymethoxyresorufin (BOMR) and octyloxymethoxyresorufin (OOMR) were purchased from Invitrogen (Carlsbad, CA). The neonicotinoid insecticides thiacloprid and the imidacloprid as well as 7-n-pentoxy coumarin (PC) were obtained from Bayer AG (Monheim, Germany).

2.2. Insects and dissection

Adult worker honey bees, *Apis mellifera* (Hymenoptera: Apidae) of mixed age were collected from queen-right colonies maintained at Bayer AG (Monheim, Germany). The health status of the colony was frequently checked by visual inspection. The colonies had not received chemical treatments for at least four weeks before testing.

Unless otherwise noted, adult worker honey bees were randomly collected from combs of the honey supper and transported to the laboratory. Honey bees were briefly anaesthetized with CO_2 and the venom gland sting complex was removed by holding the abdomen with tweezers and pulling out the sting and attached venom sac (Fig. 1). Subsequently the dissected abdomens (as well as thoraces and heads) were either immediately used or frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.3. Preparation of microsomal membranes

Dissected abdomens, heads or thoraces were homogenized in 10 mL ice-cold homogenization buffer (0.084 M Na_2HPO_4, 0.016 M KH_2PO_4, 1 mM EDTA, 1 mM DTT, 200 mM sucrose, pH 7.6) per g fresh weight with a Potter S (Sartorius) at 800 rotations min^{-1} and 10 strokes. The homogenate was filtered through three layers of cheesecloth to remove remaining chitin fragments and afterwards centrifuged at 5000 ×*g* for 5 min at 4 °C (Centrifuge 5810 R, Eppendorf) to remove cell fragments. The resulting supernatant was centrifuged at 15,000 x*g* for 20 min at 4 °C (COL90K, Beckmann, Rotor 70Ti) to separate mitochondria. Microsomal membranes were isolated by high-speed sedimentation of the supernatant at 100,000 x *g* (COL90K, Beckmann, Rotor 70Ti) for 1 h at 4 °C. The microsomal pellet was resuspended in 0.1 M sodium phosphate buffer (0.084 M Na_2HPO_4, 0.016 M KH_2PO_4, pH 7.6) using a tissue grinder and diluted to 2 mg protein mL^{-1}. The amount of protein was determined according to Bradford (1976). Rat liver microsomes (male, Wistar Han) were purchased at BD Gentest (Woburn, MA, USA) and stored according to the manufacturer's instructions. Rat liver microsomes served as positive control for cytochrome P450 activity measurement and inhibition studies.

2.4. Cytochrome P450 measurement (incl. CO difference spectra)

The functional activity of microsomal P450s was confirmed using a range of coumarin- and resorufin-based fluorogenic artificial model substrates (Zimmer et al., 2014). The enzymatic assay was conducted according to the method described by Manjon et al. (2018). Fluorescence was measured in a Spectra-Max M2 photometer (Molecular Devices) at the respective excitation/emission wavelength after 30 min incubation (Manjon et al., 2018).

The functionality of microsomal P450s was determined by carbon monoxide difference spectra (Omura and Sato, 1964). The CO difference spectra was recorded with a double-beam photometer (SPECORD® 250 PLUS, Analytik Jena AG) according to Guengerich et al. (2009). The samples contained 0.5 mg protein mL^{-1} diluted in 0.1 M sodium

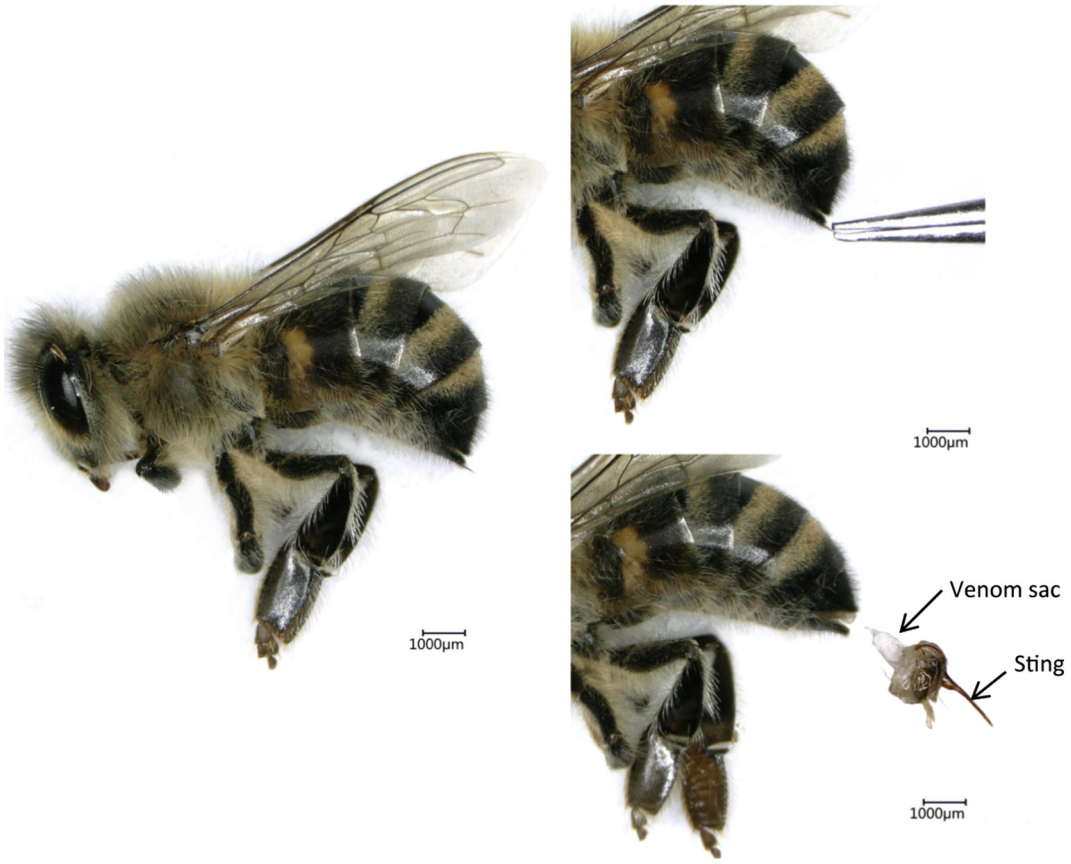

Fig. 1. Removal of the sting venom gland complex including the venom sac of a CO_2 anaesthetized honey bee using a pair of tweezers.

phosphate buffer (0.084 M Na_2HPO_4, 0.016 M KH_2PO_4, pH 7.6).

2.5. Partial purification of phospholipase A_2 by FPLC

Size exclusion chromatography was performed on 500 µL honey bee venom containing 1 mg venom protein buffered in 0.1 M NaCl (pH 7.6). Protein separation by FLPC (Äkta Avant) was carried out under following conditions: Amersham BioScience Column Superdex 200 10/300 GL, flow rate (mL-/min): 0.5, column pressure limit (Mpa): 1500, averaging time UV: 5.10, empty loop (mL): 1, eluate fraction size (mL): 1, length of elution (CV): 1.25. Fifty µL of each fraction obtained from size exclusion chromatography was tested for its inhibition potential on rat liver microsomes measuring the O-dealkylation of 7-methoxy-4-trifluoromethylcoumarin.

The molecular weight of proteins from selected FLPC fractions was separated by a gradient SDS-Page according to manufacturer instructions (NuPAGE®Novex® Bis-Tris Mini Gel, Thermo Fisher). Thirteen µL of the purified protein fraction, 5 µL NuPAGE®LDS sample buffer and 2 µL NuPAGE®reducing agent (Thermo Fisher) were incubated at 70 °C for 10 min before loading onto the gel. The NuPAGE® MES SDS running buffer (Thermo Fisher) was prepared according to the manufacturer's instructions and filled into the electrophoresis chamber (XCell *SureLock*^{Tm} Mini-Cell, Invitrogen). The samples were loaded onto the gel and during electrophoresis the chamber was stored on ice. A protein ladder (Precision Plus Protein All Blue Standards 10–250 kDa, Bio-Rad) loaded onto the gel and served as a molecular weight marker. The fractions were separated at 200 V for 35 min, the gel rinsed and stained with Coomassie brilliant blue (Imperial Protein Stain, Thermo Scientific) for one hour and destained over night while gently shaking.

2.5.1. Phospholipase A_2 activity measurement and inhibition

The Phospholipase A_2 activity was determined using the EnzChek® Phospholipase A_2 assay kit (Invitrogen) according to the manufacturer's instructions.

PLA_2 activity was inhibited with the known inhibitor manoalide (Enzo LifeScience). A serial dilution starting with 60 µM manoalide dissolved in DMSO was prepared and 10 µL of the inhibitor solution was added to 40 µL PLA_2 fractions and 50 µL substrate-liposome-mixture. The remaining PLA_2 activity at different inhibitor concentrations was determined with the EnzChek® Phospholipase A_2 assay kit (Invitrogen) according to the manufacturer's instructions.

2.6. Neonicotinoid depletion assay

To assess the metabolism of thiacloprid and imidacloprid by microsomal P450s, honey bee native microsomes (1 mg protein mL^{-1}) were incubated with 10 µM substrate in the presence of a NADPH-regeneration system (Promega: 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, 0.4 U mL^{-1} glucose-6-phosphte dehydrogenase) at 30 °C in a total assay volume of 200 µL for 0, 30 and 60 min. Native honey bee microsomes incubated without NADPH served as a control. The reaction was stopped at the respective time point by adding 800 µL ice-cold acetonitrile. The samples were centrifuged for 10 min at 3000 $\times g$ and the supernatant analyzed for the presence of formed 5`-hydroxy metabolite by tandem mass spectrometry according to the method described by Manjon et al. (2018).

2.7. Illustration of the venom sac preparation

Pictures illustrating the preparation of the venom gland sting complex from *A. mellifera* were taken with a digital microscope at 20-fold magnification (Keyence VHX-5000).

2.8. Data analysis

Data analysis was performed with GraphPad Prism v7 software (GraphPad Software Inc.). Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Data are presented as mean values ± standard deviation (SD). Statistical details of experiments (value of n, precision measures and definitions of significance) are provided in figure legends, if appropriate. Statistically significant differences ($P < .01$) between neonicotinoid hydroxylation capacity at different time intervals by honey bee microsomes was analyzed by one-way analyses of variance (ANOVA) with post-hoc testing (Tukey's HSD test).

3. Results and discussion

3.1. Preparation of functional microsomes from honey bee abdomen

The problems associated with the isolation of functional microsomes from worker bees were approached by first preparing microsomal fractions from separated worker bee heads, thoraces and abdomens, respectively. The functional activity of microsomal P450s in these fractions was assayed with three coumarin-based fluorogenic model substrates (Fig. 2). Microsomes prepared from thoraces and abdomens virtually lacked P450 activity, whereas head microsomal preparations showed a significant response, particularly using BFC as a substrate. The experiment confirmed previous observations that microsomes prepared from worker bee abdomens lack P450 activity (Gilbert and Wilkinson, 1974, 1975; Yu et al., 1984).

A major difference between honey bees and other insects is the abdominal presence of a venom gland sting complex for defensive purposes (Nouvian et al., 2016). The honey bee venom gland is a rather simple structure with secretory cells producing the venom which is collected in a reservoir (Bridges and Owen, 1984). The honey bee venom is well characterised and consists of various biologically active molecules such as peptides and proteins with diverse effects (Banks and Shipoloni, 1986; Dotimas and Hider, 1987; Peiren et al., 2005). Even though none of the bee venom components has been shown to directly inhibit cytochrome P450s, we decided to remove the abdominal venom gland sting complex prior to tissue homogenization (Fig. 1), thus resulting in highly active microsomal membranes from worker bee abdomens when incubated with a fluorogenic coumarin-based model substrate (Fig. 3A). Our finding strongly indicated that the previously described factor leading to the inhibition of microsomal P450 activity in honey bee gut preparations is part of the venom sac rather than located in the midgut (Gilbert and Wilkinson, 1974, 1975). In order to investigate this we incubated commercial rat liver microsomes with a serial dilution of honey bee venom sac equivalents and detected a strong concentration-dependent inhibition of P450 activity in a standard fluorescence-based assay (Fig. 3B), thus confirming the presence of an inhibitor in venom sac fluid.

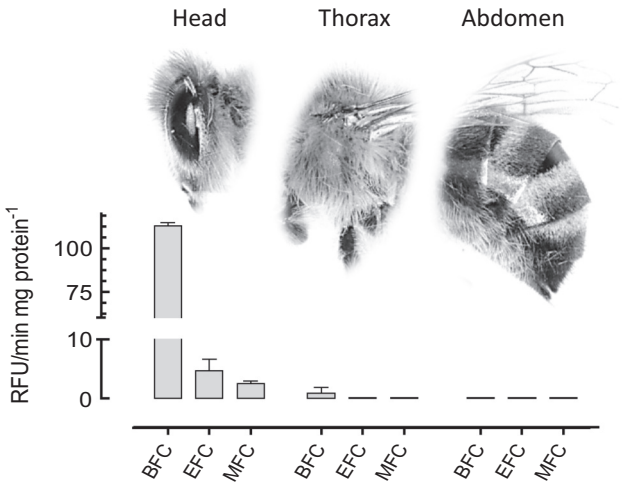

Fig. 2. Cytochrome P450 activity in microsomal preparations of different body parts of *Apis mellifera*. Enzyme activity is given in relative fluorescence units (RFU) based on incubations with different model substrates: 7-benzyloxy-4-trifluoromethyl coumarin (BFC), 7-ethoxy-4-trifluoromethyl coumarin (EFC) and 7-methoxy-4-trifluoromethyl coumarin (MFC). Data are mean values ± SD ($n = 5$).

3.2. Isolation and characterization of the inhibitor

In a next step we subjected collected honey bee venom fluid to FPLC and fractionated separated proteins by size exclusion chromatography. The elution profile followed by absorbance measurement at 280 nm revealed two major peaks and in total 35 fractions were collected (Fig. 4A). Aliquots of each FLPC fraction were tested for their inhibitory action on P450 activity in rat liver microsomes and a number of fractions, particularly fraction 16–18, corresponding to one of the major peaks showed high inhibitory potential (Fig. 4B). The other major protein fraction detected does not show any inhibitory activity. Subsequently the P450 activity inhibiting fractions were subjected to SDS-PAGE for protein separation and molecular weight determination. Those fractions with the highest P450 inhibitory activity (#16 and #17) showed two prominent protein bands in the molecular weight range between 15 kDa and 20 kDa (Fig. 5A). Honey bee venom contains numerous proteins and peptides, but two major components, melittin and phospholipase A_2 (Schmidt, 1982). Honey bee phospholipase A_2 has been shown to consist of several isoforms showing molecular weights between approx. 16 kDa and 20 kDa (Altmann et al., 1991; Peiren et al., 2005). Marker protein assisted electrophoretic analysis revealed an apparent molecular weight of approx. 17,300 Da of the major protein of fractions 16 and 17 (Fig. 5B), *i.e.* exactly corresponding to the reported molecular weight range of honey bee phospholipase A_2 (Altmann et al., 1991; Balsinde et al., 1999).

Our thoughts on the identity of the P450 inhibiting factor were further supported by the obtained electrophoretic banding pattern and molecular weight of a purified commercial honey bee phospholipase A_2 sample (Fig. 5A).

As an additional line of evidence we measured the phospholipase A_2 activity in some FPLC fractions (#16–17) showing inhibitory action on microsomal membrane preparations; this was done in comparison to one of the fractions devoid of P450 inhibitory activity (Fig. 6A). The presence of high phospholipase A_2 activity in those FPLC fractions showing P450 inhibitory activity strongly supports the fact that phospholipase A_2 is the "potent intracellular endogenous inhibitor of microsomal oxidation" originally described by Gilbert and Wilkinson (1974). The finding is additionally supported by the inhibition of the measured phospholipase A_2 activity in FPLC fraction #16 by manoalide, a known inhibitor of the enzyme (Glaser and Jacobs, 1986). The I_{50}-value of manoalide was determined at 1.2 µM (CI95% 0.40–3.7) as shown in Fig. 6B.

Phospholipase A_2 belongs to an important enzyme family involved in the degradation of fatty acids by hydrolyzing 2-acyl bonds of glycerophospholipids to lysophospholipids and arachidonic acid (Habermann, 1972; Dennis, 1991). Thus, it is highly likely that the observed "inhibition" of microsomal P450 activity is not directly mediated on the enzyme level, but by disintegrating microsomal membranes, thus affecting the proper function of these membrane-bound P450s. Removal of the abdominal venom gland sting complex (containing phospholipase A_2) before tissue homogenization resulted in functional microsomes from worker honey bee abdomens. Honey bees need to be properly anaesthetized before dissection, otherwise the segregation of the venom would result in inactive microsomal preparations as noticed in earlier trials, so removal of the venom gland sting complex is best done with the visual support of a binocular.

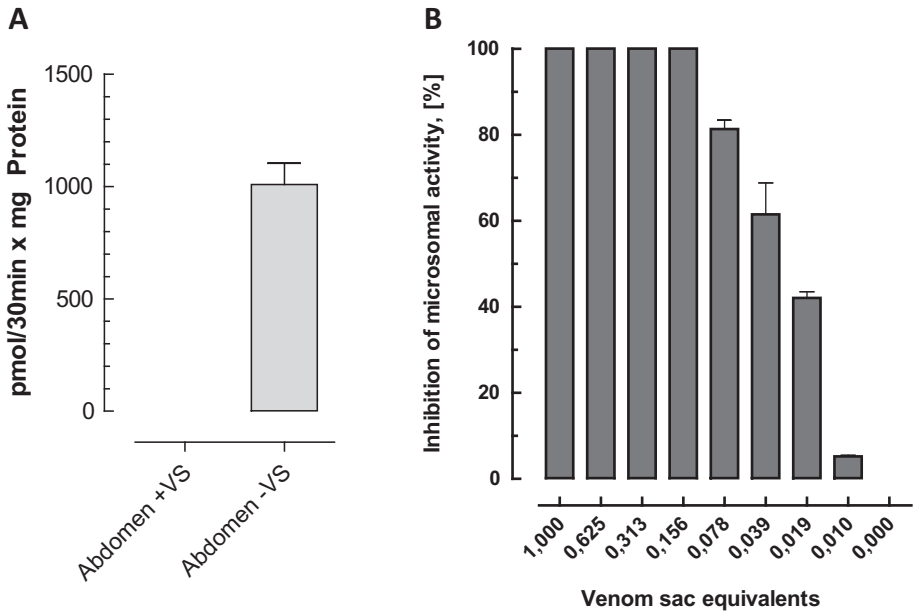

Fig. 3. (A) Cytochrome P450 activity using benzoxyfluorocoumarin as a substrate of honey bee micrsomes prepared from abdomens with (+*VS*) and without (-*VS*) venom sac. (B) Microsomal inhibition of 7-ethoxycoumarin O-deethylase activity in rat liver microsomes by serial dilutions of honey bee venom sac equivalents. Data are mean values ± SD (n = 3).

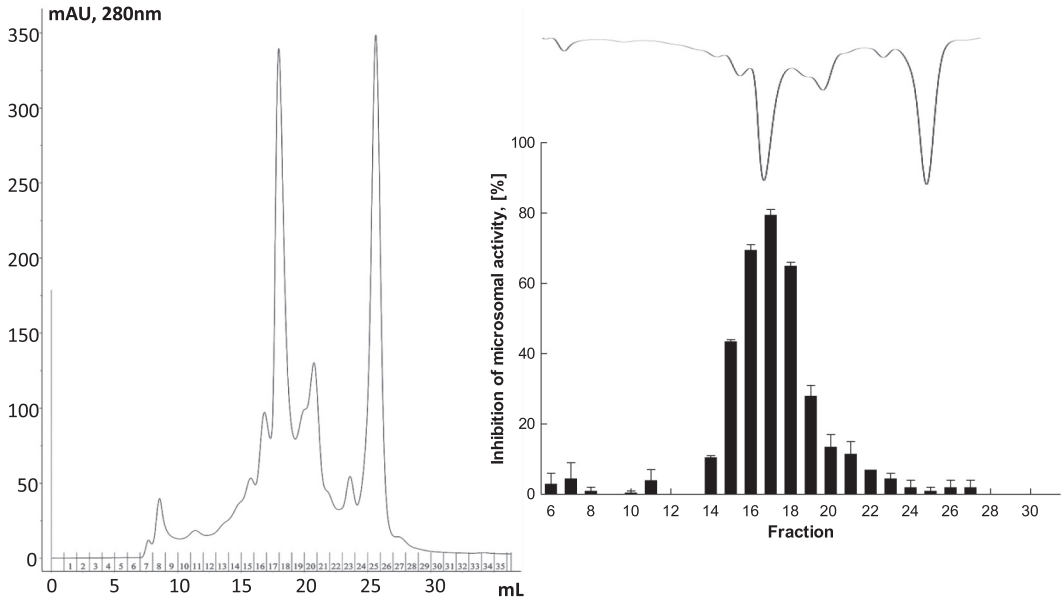

Fig. 4. Elution profile of honey bee venom sac proteins (1 mg) after size exclusion chromatography on Superdex S200 10/300 GL. The absorbance (mAU) was measured at 280 nm and 1 mL fractions were collected. Aliquots were subjected to a rat microsomal inhibition assay using 7-ethoxycoumarin.

3.3. Biochemical characterization of microsomal honey bee P450s

The presence of functional P450s was confirmed by determining the CO difference spectra of honey bee microsomes prepared in the absence (-*VS*) and presence (+*VS*) of the venom gland sting complex (Fig. 7A and B, respectively). Commercial microsomal fractions from rat liver served as a positive control (Fig. 7C). The bee microsomal fraction, prepared from abdomens without the venom gland sting complex, shows a similar profile with a characteristic peak at 450 nm (Omura and Sato, 1964) (Fig. 7A); however, in microsomes prepared from abdomens with the venom gland sting complex P450s are largely non-functional as shown by a prominent absorbance maximum at 420 nm (Fig. 7B).

The catalytic activity of honey bee microsomal P450s was assayed with a range of coumarin- and resorufin-based fluorogenic artificial model substrates. Honey bee microsomal P450s prepared from dissected abdomens (-*VS*) showed a strong preference for coumarin based derivates over resorufins, whereas microsomes prepared from abdomens without prior dissection (+*VS*) no significant turnover of model substrates (Fig. 8). The highest specific activity was measured with 7-benzyloxy-4-trifluoromethyl coumarin, BFC (126 ± 3.94 pmol product/min mg protein^{-1}) followed by the O-alkylated substrates 7-n-pentoxy coumarin (PC) and 7-ethoxy-4-trifluoromethyl coumarin (EFC). The preference of honey bee microsomal P450s to metabolize bulkier substrates such as BFC and 7-benzyloxymethoxy-4-trifluoromethyl coumarin (BOMFC) as well as the preference for coumarin based substrates confirms previous observations on substrate profiles of functionally expressed CYP9Q-enzymes (Manjon et al., 2018). The ethoxycoumarin-O-deethylase activity recently described by Yu et al. (1984) in microsomes prepared from isolated honey bee midguts was also present in microsomal preparations of complete abdomens investigated in this study.

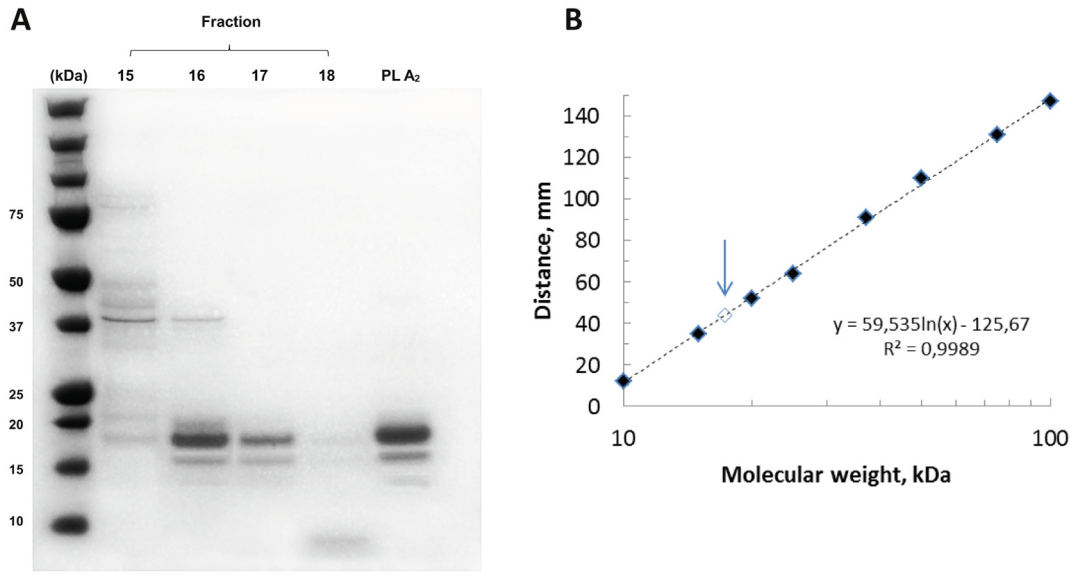

Fig. 5. (A) SDS PAGE of selected FPLC fractions of eluted honey bee venom sac proteins exhibiting the highest inhibitory activity on rat microsomal O-deethylation activity (EC). Molecular weight marker proteins are shown on the left, whereas a purified commercial sample of bee venom phospholipase A_2 (PLA_2) is shown on the right lane. (B) The molecular weight determination (indicated by an arrow) of the eluted protein is shown on the right.

3.4. Variation of microsomal P450 activity among bee hives

The variation in P450 activity among bees collected in different hives was assessed by measuring the capacity of O-dearylation of BFC by respective microsomal preparations from mass homogenates of pooled worker abdomens. The substrate was chosen, because honey bee microsomal preparations described above showed by far the highest activity with BFC. In total we sampled nine hives located almost next to each other, but the turnover of BFC by microsomal P450s was highly variable among hives (Fig. 9A). The honey bees used for the preparation of microsomal fractions were randomly collected from combs placed in the honey super, so they were likely of mixed age, possibly explaining the variation in activity. The collection and preparation of microsomes from a higher number of bees may result in less variation and more consistent activity results; however this needs to be shown in future experiments. Recently it has been demonstrated that forager bees exhibit elevated levels of P450 and glutathione-S transferases activity when compared to younger bees that are in charge of in-hive tasks (Smirle and Winston, 1987; Smirle and Robinson, 1989). To further investigate this observation, microsomal fractions from honey bees ($n = 10$) collected in five different hives each from the honey supper and caught at the flight hole were prepared and screened for P450 activity using BFC. While only two out of five microsomal fractions prepared from individual in-hive bees showed P450 activity, all microsomal preparations from forager bees caught at the flight hole displayed P450 activity (Fig. 9B). The results principally confirm earlier observations by Smirle and Winston (1987), suggesting that foragers collected at the flight hole of the hive should be preferred for the preparation of microsomal membranes.

As the preparation of honey bee microsomes takes some time and is seasonally restricted, we additionally examined the storage stability of honey bee microsomes at −80 °C. Our experiments revealed that frozen microsomal fractions are stable for at least 6 month after freezing (Fig. 10) and thus offering the possibility for their use in biochemical studies off season.

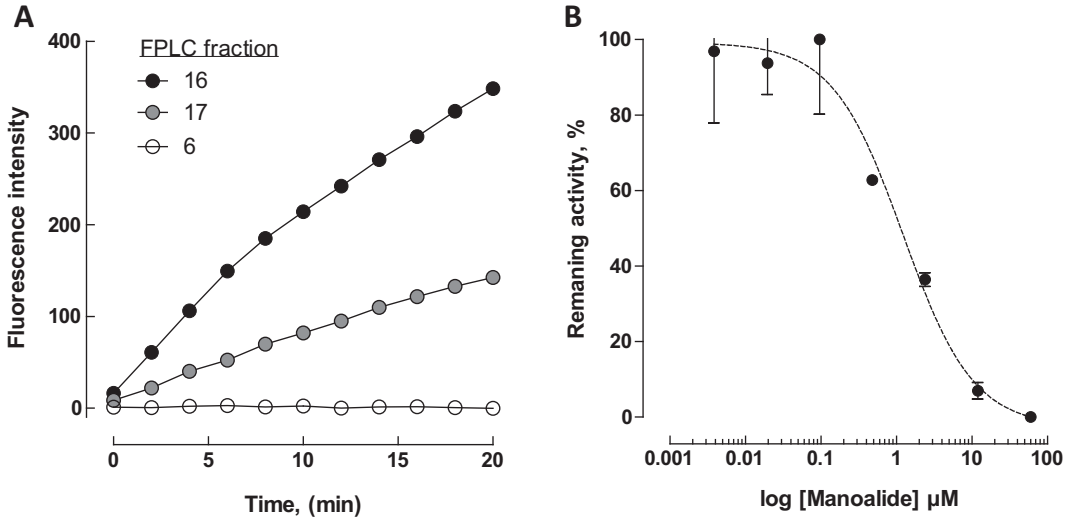

Fig. 6. (A) Measurement of phospholipae A_2 activity in FPLC fractions of honey bee venom collected after size exclusion chromatography on Superdex S200. (B) Inhibition by manoalide of honey bee phospholipase A_2 activity present in FPLC fraction #16.

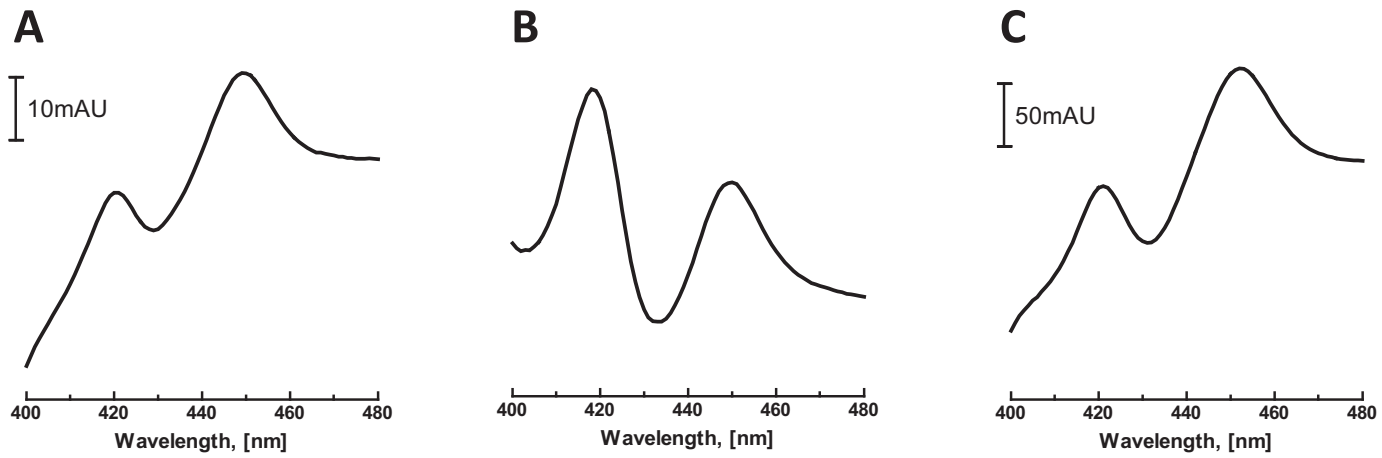

Fig. 7. CO-binding difference spectra recorded from microsomal preparations. (A) honey bee abdomens, venom sac removed; (B) honey bee abdomens, venom sac included; (C) rat liver.

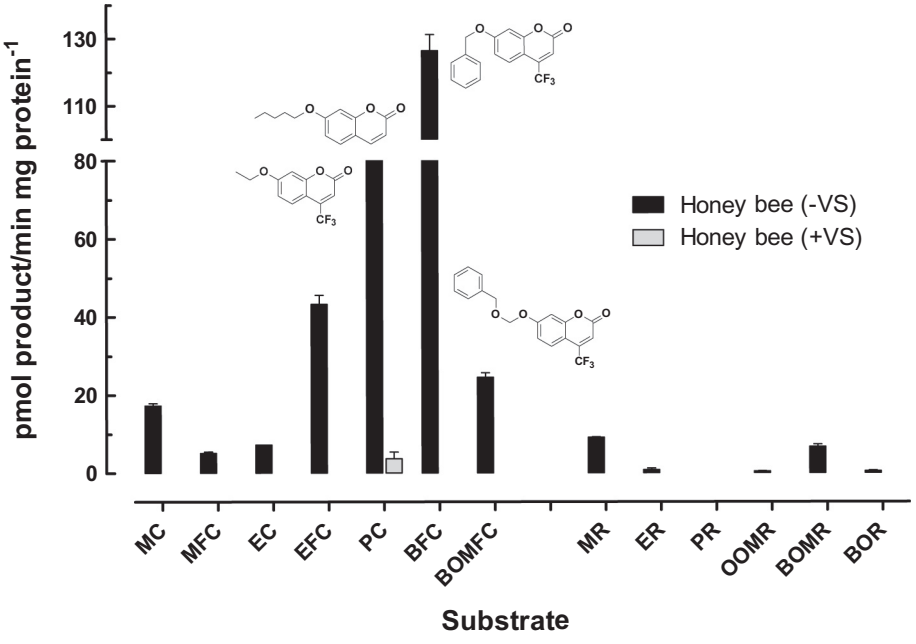

Fig. 8. Metabolism (O-dealkylation/−dearylation) of different fluorogenic coumarin and resorufin substrates by microsomal preparations obtained from honey bee (± *VS*, venom sac). Data are mean values ± SD (n = 3).
Abbreviations: BFC, 7-benzyloxy-4-trifluoromethyl coumarin; MFC, 7-methoxy-4-trifluoromethyl coumarin; EFC, 7-ethoxy-4-trifluoromethyl coumarin; BOMFC, 7-benzyloxymethoxy-4-trifluoromethyl coumarin; PC, 7-n-pentoxy coumarin; EC, 7-ethoxy coumarin; MC, 7-methoxy coumarin; BOMR, 7-benzyloxymethoxyresorufin; ER, 7-ethoxyresorufin; BOR, 7-benzyloxyresorufin; MR, 7-methoxyresorufin; OOMR, octyloxymethoxyresorufin; PR, 7-n-pentoxyresorufin.

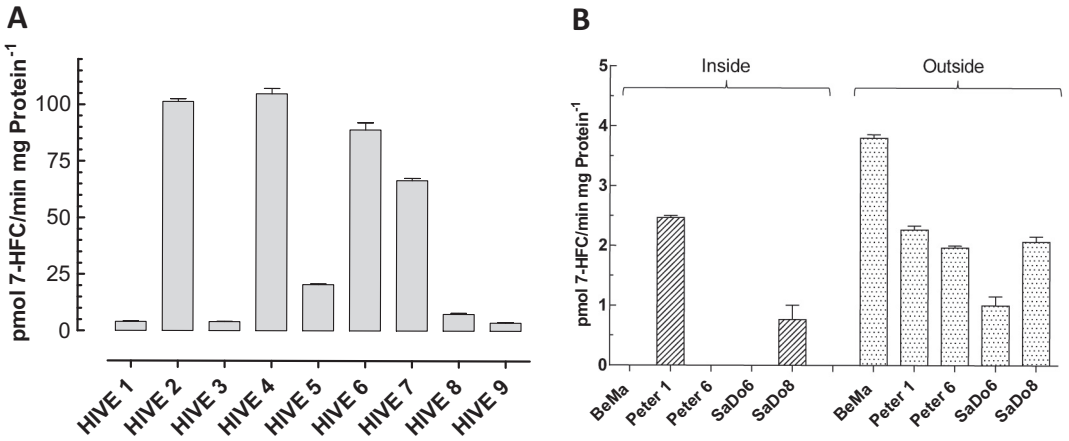

Fig. 9. (A) Variation in cytochrome P450 activity of microsomal preparations of mass homogenates of honey bees (n = 15) collected from combs of different hives (same day) using 7-benzyloxy-4-trifluoromethyl coumarin (BFC) as a substrate. (B) Variation of microsomal BFC O-dearylation activity of honey bees (n = 10) collected inside (combs) and outside (entrance hole) of a different set of hives. Data are mean values ± SD (n = 3).

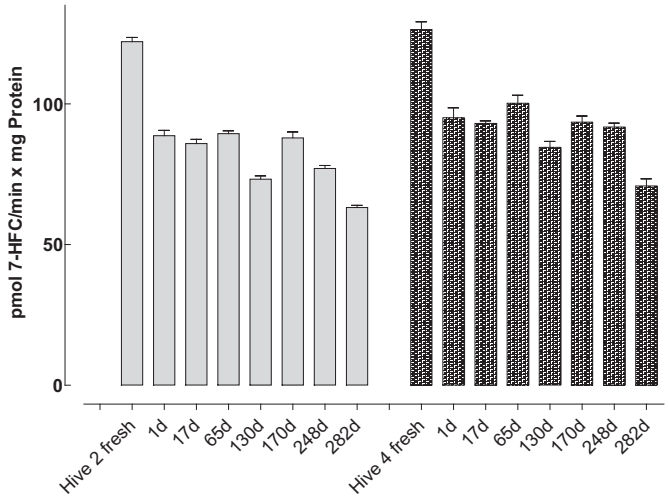

Fig. 10. Stability at −80 °C of microsomal preparations of honey bees collected from two different hives. The microsomal activity was measured after thawing at different elapsed time intervals using 7-benzyloxy-4-trifluoromethyl coumarin (BFC) as a substrate. Data are mean values ± SD (n = 3).

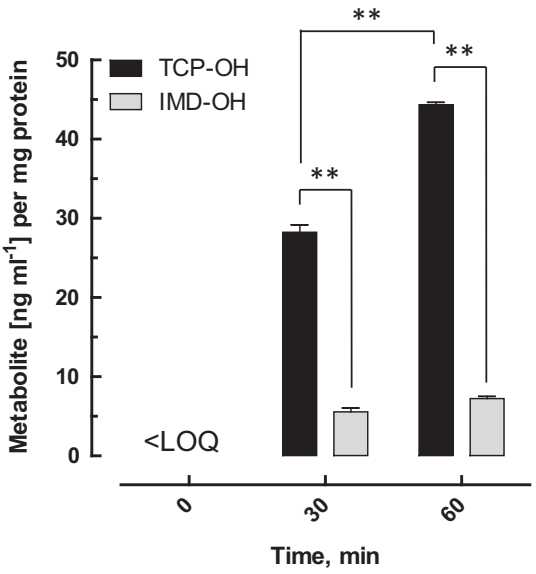

Fig. 11. Hydroxylation of thiacloprid (TCP) and imidacloprid (IMD) at different elapsed time intervals by microsomes prepared from honey bee abdomens after removal of the sting venom sac complex. Microsomes incubated without NADPH were inactive (data not shown). Data are mean values ± SD (n = 3); ** (significantly different at $P < .01$). LOQ = Limit of quantitation.

3.5. Microsomal P450s degrade neonicotinoids by 5`-hydroxylation

Very recently it has been demonstrated that enzymes belonging to the CYP9Q-subfamily are key determinants mediating bee sensitivity towards neonicotinoid insecticides by rapidly hydroxylating *N*-cyano-substituted, but not *N*-nitro-substituted compounds (Manjon et al., 2018). Therefore we incubated honey bee microsomal preparations with both imidacloprid and thiacloprid to investigate the speed and level of neonicotinoid hydroxylation *in vitro*. Indeed thiacloprid was more rapidly hydroxylated than imidacloprid as shown by LC-MS/MS analysis of the respective hydroxylated metabolites at different elapsed time intervals (Fig. 11). The observed differences concerning the hydroxylation of thiacloprid and imidacloprid are highly significant ($P < .01$; ANOVA with post-hoc Tukey test). Interestingly no significant increase in the level of imidacloprid hydroxylation was observed between 30 min and 60 min, whereas the levels of hydroxylated thiacloprid significantly ($P < .001$) increased between 30 min and 60 min (Fig. 11). It has been recently demonstrated *in vivo* that hydroxylated thiacloprid is a main metabolite in honey bees upon topical application (Zaworra et al., 2019), a finding confirmed and reflected in this study by the presence of the same metabolite in microsomal incubations *in vitro*, most likely driven by CYP9Q3 as recently demonstrated by Manjon et al. (2018). CYP9Q3 has been demonstrated to show a clear preference for *N*-cyano-substituted neonicotinoids such as thiacloprid rather than *N*-nitro-substituted neonicotinoids such as imidacloprid (Manjon et al., 2018). This is confirmed by the results of microsomal oxidation presented here.

4. Conclusions

In this study we shed light on an old problem linked to the preparation of functional microsomal fractions from honey bee abdomens. Simply the removal of the venom gland sting complex resulted in the preparation of highly active microsomes from worker bee abdomen. Moreover we provided compelling evidence that bee venom protein phospholipase A_2 is the "potent intracellular endogenous inhibitor of microsomal oxidation" originally described by Gilbert and Wilkinson (1974), most likely by disintegrating microsomal membranes. Although the presented method still requires some labour with regard to the dissection of the venom gland sting complex, it is less laborious - and possibly more effective - than isolating intact midguts or fatbodies for the investigation of the oxidative metabolism of xenobiotics. The study provided furthermore a characterization of the fluorogenic substrate specificity of honey bee microsomes to be used in future biochemical assays. The highest specific activity of microsomal honey bee P450s has been shown for BFC.

The functional status of the prepared microsomes was further validated by the detoxification capacity of thiacloprid mediated by its hydroxylation. Thus demonstrating that our work provides access to a new and simple *in vitro* tool to study honey bee phase 1 metabolism of xenobiotics utilising the entire range of microsomal cytochrome P450s.

Competing interests

The authors have declared that no competing interests exist.

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Chapter 3

Unravelling the molecular determinants of bee sensitivity to neonicotinoid insecticides

Authors: Cristina Manjon*, Bartlomiej J. Troczka*, Marion Zaworra*, Katherine Beadle, Emma Randall, Gillian Hertlein, Kumar Saurabh Singh, Christoph T. Zimmer, Rafael A. Homem, Bettina Lueke, Rebecca Reid, Laura Kor, Maxie Kohler, Juergen Benting, Martin S. Williamson, T.G. Emyr Davies, Linda M. Field, Chris Bass and Ralf Nauen

* These authors contributed equally

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Report

Current Biology

Unravelling the Molecular Determinants of Bee Sensitivity to Neonicotinoid Insecticides

Highlights

- Honeybees and bumble bees show variation in sensitivity to different neonicotinoids
- Variation in bee sensitivity to neonicotinoids does not reside at the target site
- Cytochrome P450s of the CYP9Q subfamily determine bee sensitivity to neonicotinoids
- CYP9Q genes are highly expressed in bee Malpighian tubules and the brain

Authors

Cristina Manjon, Bartlomiej J. Troczka, Marion Zaworra, ..., Linda M. Field, Chris Bass, Ralf Nauen

Correspondence

c.bass@exeter.ac.uk (C.B.), ralf.nauen@bayer.com (R.N.)

In Brief

N-cyanoamidine neonicotinoid insecticides are much less toxic to honeybees and bumble bees than *N*-nitroguanidine compounds. Manjon et al. show that this results from differences in their efficiency of metabolism by cytochrome P450s of the CYP9Q subfamily, demonstrating their role as key determinants of bee sensitivity to this insecticide class.

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April 2, 2018
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CellPress

Current Biology
Report

Unravelling the Molecular Determinants of Bee Sensitivity to Neonicotinoid Insecticides

Cristina Manjon,[1,5] Bartlomiej J. Troczka,[2,5] Marion Zaworra,[1,4,5] Katherine Beadle,[3] Emma Randall,[3] Gillian Hertlein,[1] Kumar Saurabh Singh,[3] Christoph T. Zimmer,[3,6] Rafael A. Homem,[2] Bettina Lueke,[1] Rebecca Reid,[2] Laura Kor,[2] Maxie Kohler,[1] Jürgen Benting,[1] Martin S. Williamson,[2] T.G. Emyr Davies,[2] Linda M. Field,[2] Chris Bass,[3,7,*] and Ralf Nauen[1,*]

[1]Bayer AG, Crop Science Division, Alfred Nobel-Strasse 50, 40789 Monheim, Germany
[2]Department of Biointeractions and Crop Protection, Rothamsted Research, Harpenden, UK
[3]College of Life and Environmental Sciences, Biosciences, University of Exeter, Penryn Campus, Penryn, Cornwall, UK
[4]Institute of Crop Science and Resource Conservation, Rheinische Friedrich-Wilhelms University Bonn, 53115 Bonn, Germany
[5]These authors contributed equally
[6]Present address: Syngenta Crop Protection, Werk Stein, Schaffhauserstrasse, Stein CH4332, Switzerland
[7]Lead Contact
*Correspondence: c.bass@exeter.ac.uk (C.B.), ralf.nauen@bayer.com (R.N.)
https://doi.org/10.1016/j.cub.2018.02.045

SUMMARY

The impact of neonicotinoid insecticides on the health of bee pollinators is a topic of intensive research and considerable current debate [1]. As insecticides, certain neonicotinoids, i.e., *N*-nitroguanidine compounds such as imidacloprid and thiamethoxam, are as intrinsically toxic to bees as to the insect pests they target. However, this is not the case for all neonicotinoids, with honeybees orders of magnitude less sensitive to *N*-cyanoamidine compounds such as thiacloprid [2]. Although previous work has suggested that this is due to rapid metabolism of these compounds [2–5], the specific gene(s) or enzyme(s) involved remain unknown. Here, we show that the sensitivity of the two most economically important bee species to neonicotinoids is determined by cytochrome P450s of the CYP9Q subfamily. Radioligand binding and inhibitor assays showed that variation in honeybee sensitivity to *N*-nitroguanidine and *N*-cyanoamidine neonicotinoids does not reside in differences in their affinity for the receptor but rather in divergent metabolism by P450s. Functional expression of the entire CYP3 clade of P450s from honeybees identified a single P450, CYP9Q3, that metabolizes thiacloprid with high efficiency but has little activity against imidacloprid. We demonstrate that bumble bees also exhibit profound differences in their sensitivity to different neonicotinoids, and we identify CYP9Q4 as a functional ortholog of honeybee CYP9Q3 and a key metabolic determinant of neonicotinoid sensitivity in this species. Our results demonstrate that bee pollinators are equipped with biochemical defense systems that define their sensitivity to insecticides and this knowledge can be leveraged to safeguard bee health.

RESULTS AND DISCUSSION

Bees carry out vital ecosystem services by pollinating wild plants and economically important crops but, in doing so, are exposed to a wide variety of natural and synthetic xenobiotics (including pesticides) [6]. Understanding the molecular defense systems that bees use to protect themselves from these potential toxins and their effectiveness and specificity provides important knowledge that can be used to avoid negative off-target effects [7].

Previous studies have demonstrated that honeybees exhibit marked differences in their sensitivity to different pesticides. Indeed, certain compounds display such low acute toxicity to bees that they are used as in-hive treatments by beekeepers against parasitic *Varroa* mites [6]. This differential sensitivity extends to neonicotinoid insecticides, with honeybees exhibiting profound differences in their sensitivity to N-nitroguanidine and N-cyanoamidine neonicotinoids [2]. In this study, we used imidacloprid and thiacloprid as exemplars of each class and first examined whether this differential sensitivity extends to bumble bees (*Bombus terrestris*), the second-most economically important bee pollinator species worldwide. In both contact and oral bioassays, significant (> 500-fold) differences were observed in the sensitivity of bumble bees to the two compounds (Figure 1A, Table S1). Based on these results and previous data for honeybees [9, 10], imidacloprid is categorized as "highly toxic" to both bumble bees and honeybees, according to the U.S. Environmental Protection Agency (EPA) (Figure 1A) [8]. In contrast, thiacloprid is categorized as "slightly toxic" or "practically non-toxic" to both bee species depending on the route of exposure (Figure 1A) [10].

The molecular basis of the differences in sensitivity of bees to these neonicotinoids could reside in differences in their affinity for the target site, the nicotinic acetylcholine receptor (nAChR), or from differences in the speed and efficiency of their metabolism. To examine the role of the former in intrinsic bee tolerance to thiacloprid, we carried out radioligand binding assays using honeybee and bumble bee head membrane preparations, an enriched source of nAChRs, using tritiated imidacloprid and examined the displacement of [^{3}H]-imidacloprid by both

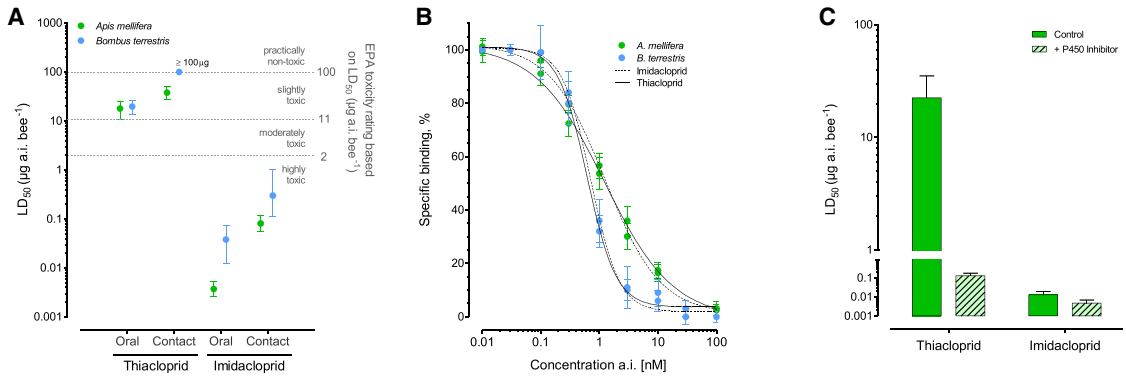

Figure 1. Toxicodynamics and Pharmacokinetics of Neonicotinoid Sensitivity in Two Bee Species

(A) LD_{50} values for imidacloprid and thiacloprid upon oral and topical application in *A. mellifera* and *B. terrestris*. Sensitivity thresholds are depicted according to EPA toxicity ratings [8]. Data for *A. mellifera* is taken from [9, 10], data for *B. terrestris* was generated in this study. Error bars display 95% CLs (n = 4).

(B) Specific binding of thiacloprid and imidacloprid to both *A. mellifera* and *B. terrestris* nAChRs. Error bars display standard deviation (n = 3).

(C) Sensitivity of *A-p-methoxy-. mellifera* to imidacloprid and thiacloprid before and after pretreatment with the insecticide synergist ABT (aminobenzotriazole). Error bars display 95% CLs (n = 3).

See also Table S1.

unlabelled imidacloprid and thiacloprid. As shown in Figure 1B, in the case of both bee species, both imidacloprid and thiacloprid bind with nM affinity, and no significant difference was seen in the specific binding of either compound at the receptor (IC_{50} of 1.2, [95% CI 0.97, 1.6] and 1.1 nM [95% CI 0.94, 1.6] for imidacloprid and thiacloprid respectively for honeybees, and IC_{50} of 0.71 [95% CI 0.62, 0.82] and 0.62 nM [95% CI 0.50, 0.77] for imidacloprid and thiacloprid for bumble bees). This finding clearly demonstrates that the differences in bee sensitivity to these two neonicotinoids is not a consequence of variation in their affinity for the nAChR.

The use of insecticide synergists that inhibit insect metabolic enzyme systems has provided evidence that one or more members of the cytochrome P450 superfamily are responsible for the tolerance of honeybees to thiacloprid [2]. Indeed, Iwasa et al. [2] demonstrated that the P450 inhibitors piperonyl butoxide (PBO), triflumizole, and propiconazole increased honeybee sensitivity to thiacloprid by 154-, 1,141- and 559-fold, respectively, but had almost no effect on honeybee sensitivity to imidacloprid. To explore this further, we used 1-aminobenzotriazole (ABT), a nonspecific suicide inhibitor of P450s that has been used widely in mammalian systems to distinguish P450-mediated metabolism from non-P450-mediated metabolism *in vitro* [11, 12]. Honeybees pretreated with ABT became > 170-fold more sensitive to thiacloprid but only 2.7-fold more sensitive to imidacloprid (Figure 1C), supporting the view that P450s underlie the variation in the sensitivity of this bee species to these two compounds. Likewise, insecticide bioassays of bumble bees after treatment with PBO resulted in a significant 4.2-fold increase in the sensitivity of bumble bees to thiacloprid but no significant shift in sensitivity to imidacloprid (Table S1). Thus, we demonstrate that P450s also appear to be an important determinant of neonicotinoid sensitivity in bumble bees. The level of synergism we observed in bumble bees is significantly lower than that reported by Iwasa et al. [2] using the same inhibitor (see above); this may in part result from differences in methodology used (contact versus oral insecticide bioassays) and/or differences in the ability of this synergist to inhibit the relevant P450 enzymes.

Insect P450 genes fall into four major clades, and enzymes from each of these clades have been linked to insecticide resistance or to the metabolism of xenobiotics [13]. However, members of the CYP3 clade, particularly those of the CYP6 and CYP9 families, have been most frequently linked to xenobiotic detoxification across a range of insect species [13–15]. Therefore, to explore which honeybee P450(s) are responsible for thiacloprid metabolism, 27 of the 46 honeybee P450 genes, comprising the entire CYP3 clade, were individually coexpressed with house fly P450 reductase (CPR) in an insect cell line. Incubation of purified microsomal preparations containing each P450 and CPR with thiacloprid and analysis of the metabolites produced by liquid chromatography tandem mass spectrometry (LC-MS/MS) identified a single P450, CYP9Q3, as the highly efficient metabolizer of thiacloprid (primarily to 5-hydroxy thiacloprid) (Figures 2A, S1, and S2). Topical bioassays of honeybees using 5-hydroxy thiacloprid revealed reduced toxicity of this metabolite (LD_{50}-value of > 100 µg/bee) relative to the parent compound (Figure 1A). Three other P450s—CYP6AS5, CYP9Q1, and CYP9Q2—showed weak activity against thiacloprid, but this was at least > 10-fold lower than that seen for CYP9Q3 (Figure 2A). Repeating these assays using imidacloprid revealed that only CYP9Q1–3 exhibit any capacity to metabolize this compound but at much lower efficiency than exhibited for thiacloprid (Figure 2A). To provide additional evidence that CYP9Q3 is the primary honeybee P450 that confers tolerance to thiacloprid *in vivo*, we created a series of transgenic *Drosophila* lines expressing honeybee *CYP9Q1*, *CYP9Q2*, or *CYP9Q3*. Flies expressing the *CYP9Q3* transgene showed a marked (> 10-fold) and significant resistance to thiacloprid compared to control flies of the same genetic background without the transgene in insecticide bioassays (Figure 2D). Flies expressing *CYP9Q1* showed no change in sensitivity to thiacloprid compared to controls, and flies expressing *CYP9Q2* showed a significant but more modest (3.5-fold) resistance to thiacloprid (Figure 2D). In bioassays using imidacloprid, no significant differences in sensitivity were observed between flies with any of the three transgenes and control flies consistent with the low efficiency of imidacloprid

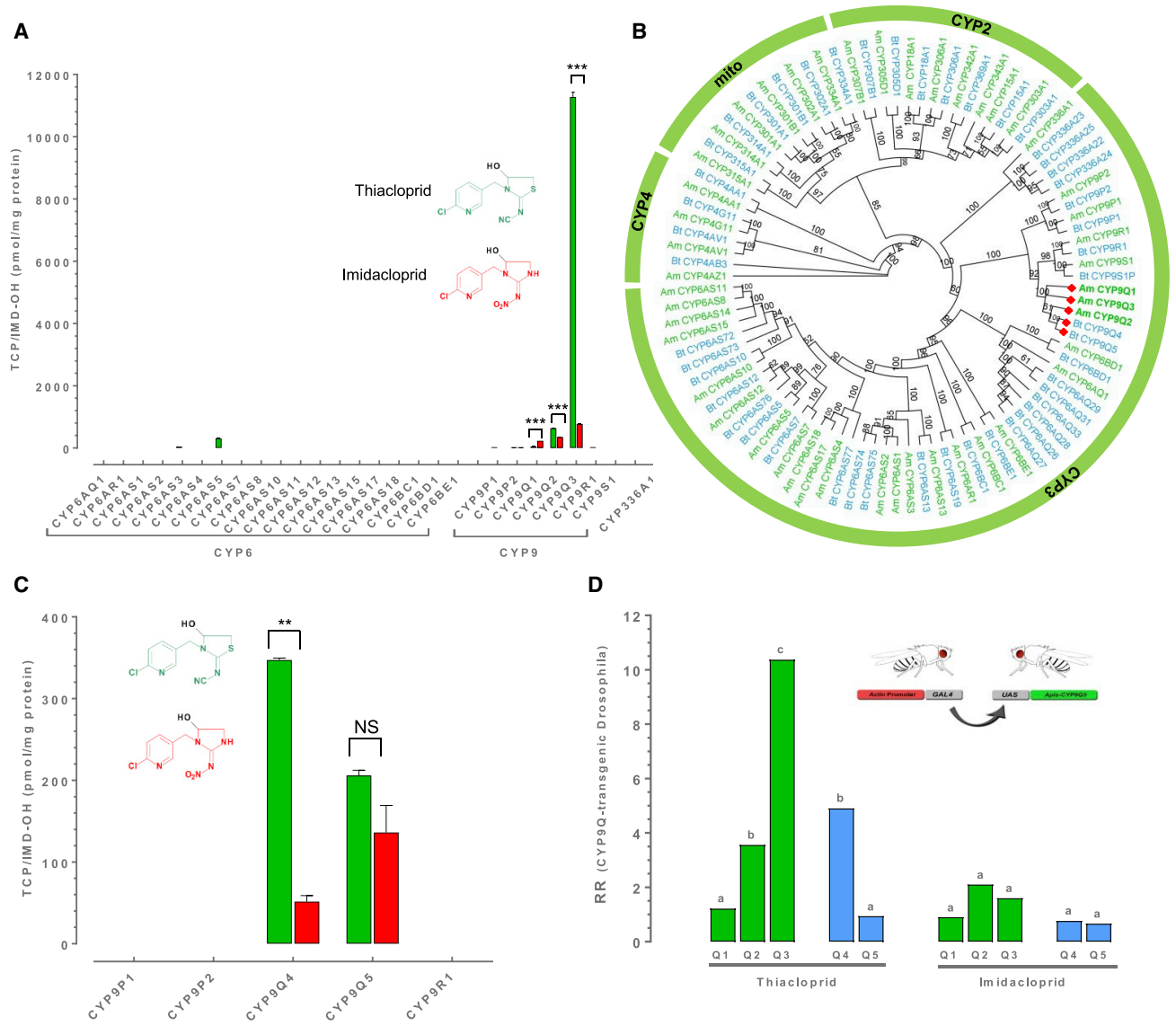

Figure 2. Identification of Neonicotinoid Metabolising P450s in Honeybee and Bumble Bee

(A and C) (A) Thiacloprid and imidacloprid hydroxylation by recombinantly expressed P450s of the *A. mellifera* CYP3 clade and (C) the CYP9 family in *B. terrestris*. The production of the hydroxy metabolite of each insecticide is displayed per mg of P450 protein (NS, not significant; **Pc < c0.01, ***Pc < c0.001; Welch's t test). Error bars display standard deviation (n = 3).

(B) Phylogenetic tree with branch bootstrap values for *A. mellifera* (green) and *B. terrestris* (blue) P450 genes. Genes are grouped according to their adscription to different P450 clades. Branches within the CYP3 clade marked with a red dot indicate the position of *A. mellifera* CYP9Qs and their closest *B. terrestris* orthologs involved in thiacloprid metabolism, as shown in (A), (C), and (D).

(D) Resistance ratio (RR) of transgenic *Drosophila* strains expressing *A. mellifera AmCYP9Q1–3* or *B. terrestris BtCYP9Q4-5* transgenes to thiacloprid and imidacloprid compared to a control line (flies of the same genetic background but without the transgene). Significance is referenced against this control line and based on non-overlapping 95% fiducial limits of LC_{50} values (n = 3).

See also Figures S1, S2, and S3.

metabolism observed *in vitro* (Figure 2D). Taken together, these results demonstrate unequivocally that the transcription of *CYP9Q3* confers strong intrinsic tolerance to thiacloprid, but not to imidacloprid.

To identify potential functional orthologs of honeybee *CYP9Q3* in the bumble bee, we compared P450s identified in the sequenced genome of *B. terrestris* [16] with *CYP9Q1–3*. Phylogenetic analysis of the *B. terrestris* CYPome revealed five candidate genes subsequently named as *CYP9P1*, *CYP9P2*, *CYP9R1*, *CYP9Q4*, and *CYP9Q5* that cluster with honeybee *CYP9Q1–3* (Figure 2B). Of these, *CYP9Q4* and *CYP9Q5* show the greatest sequence identity to honeybee *CYP9Q1–3* (Figure S3). Functional expression of these five P450s *in vitro* revealed that only CYP9Q4 and CYP9Q5 metabolize thiacloprid to its 5-hydroxy form (Figure 2C), with subsequent enzyme kinetic assays confirming that CYP9Q4 metabolizes thiacloprid more efficiently

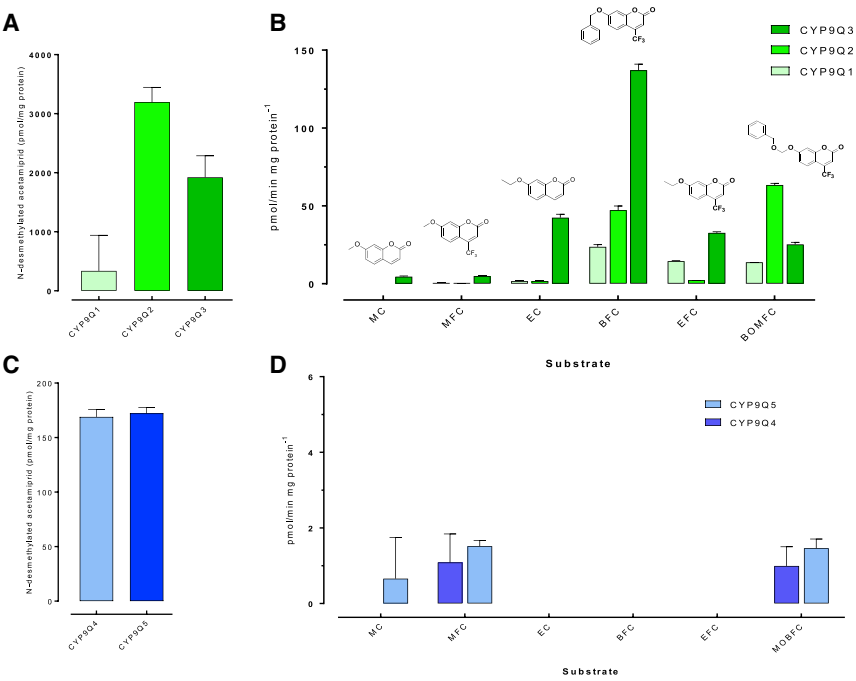

Figure 3. Metabolism of Acetamiprid and Model Substrates by Honeybee and Bumble Bee CYP9Q Subfamily P450s

(A and C) Acetamiprid N-desmethylation by recombinantly expressed CYP9Q1–3 of *A. mellifera* and (C) CYP9Q4–5 of *B. terrestris*. The production of N-desmethylated acetamiprid is displayed per mg of protein. Error bars display standard deviation (n = 3). (B and D) (B) Activity of CYP9Q1–3 and (D) CYP9Q4–5 against different fluorescent coumarin model substrates. Error bars display standard deviation (n = 3). Abbreviations: MC, 7-methoxycoumarin; MFC, 7-methoxy-4-trifluoromethyl coumarin; EC, 7-ethoxy coumarin; BFC, 7-benzyloxy-4-trifluoromethyl coumarin; EFC, 7-ethoxy-4-trifluoromethyl coumarin; BOMFC, 7-benzyloxymethoxy-4-trifluoromethyl coumarin; MOBFC, 7-p-methoxy-benzyloxy-4-trifluoro coumarin.

than CYP9Q5 (Figure S2). Further functional validation of these two P450s by expression in transgenic *Drosophila* revealed that flies expressing *CYP9Q4* exhibited significant (~5-fold) resistance to thiacloprid compared to controls, whereas flies expressing *CYP9Q5* showed no change in sensitivity (Figure 2D). As for honeybee *CYP9Q1–3*, no significant differences were observed in the sensitivity of flies expressing either *CYP9Q4* or *CYP9Q5* to imidacloprid compared to controls (Figure 2D). Thus, these findings demonstrate that members of the *CYP9Q* subfamily also confer tolerance to thiacloprid in *B. terrestris*.

To further explore the substrate specificity of CYP9Q1–5, we tested their functional activity against a range of fluorescent model substrates and acetamiprid, a second N-cyanoamidine neonicotinoid that also has low acute toxicity to honeybees and is very rapidly metabolized *in vivo* [4]. Against coumarin model substrates, honeybee CYP9Q1–3 show a preference for bulkier molecules such as BFC and BOMFC, with CYP9Q1 and CYP9Q3 both showing highest specific activity for BFC (Figure 3B). In addition, CYP9Q3 demonstrated a pattern of broader substrate specificity than the other two P450s, suggestive of a more promiscuous active site (Figure 3B). These results contrasted with bumble bee CYP9Q4 and CYP9Q5, which showed no activity against BFC and, in the case of CYP9Q4, a noticeably reduced substrate specificity with activity against just two of the model substrates tested (MFC and MOBFC) (Figure 3D). Incubation of recombinant CYP9Q1–5 with acetamiprid followed by LC-MS/MS analyses revealed that all five P450s have the capacity to metabolize this compound to N-desmethyl acetamiprid, with CYP9Q2–5 exhibiting the highest activity (Figures 3A and 3C). Thus, our data demonstrate that the rapid metabolism of acetamiprid reported *in vivo* [4] is likely mediated, at least in part, by P450s of the CYP9Q subfamily.

The CYP9Q subfamily of P450s has been implicated in the metabolism of xenobiotics previously, with honeybee CYP9Q1–3 all found to metabolize the pyrethroid tau-fluvalinate and the organophosphate coumaphos, two insecticides that show marked selectivity for mites (i.e., *Varroa*) over bees [15]. Our findings reveal that in both honey bees and bumble bees, this P450 subfamily contains potent metabolizers of certain neonicotinoid insecticides, thus explaining the low acute toxicity of thiacloprid and acetamiprid. In humans, just a handful of the 57 functional P450s are responsible for the biotransformation of most foreign chemicals; for example, CYP3A4 and CYP2D6 together are responsible for the metabolism of > 50% of clinically used drugs [17]. The finding that members of the bee CYP9Q subfamily have the capacity to metabolize compounds belonging to three different insecticide classes suggests that they may act as functional insect equivalents of these human P450s and thus are critically important in defining the sensitivity of eusocial bees to xenobiotics.

To identify the primary sites of CYP9Q-mediated detoxification, P450 expression was assessed in bee body parts and dissected tissues that are commonly involved in xenobiotic detoxification [18] by quantitative PCR (qPCR). *CYP9Q3* was expressed at high levels in the honeybee brain and Malpighian tubules (Figure 4A), the latter finding consistent with a previous study which examined expression in honeybee tissues, including the Malpighian tubules, by RNA-seq [19]. In *B. terrestris*, *CYP9Q4* and *CYP9Q5* showed marked differences in their pattern of spatial expression, with *CYP9Q4* highly expressed in the brain (> 60-fold greater than in the other tissues tested) and *CYP9Q5* expressed at relatively uniform levels in the midgut, Malpighian tubules, and brain (Figure 4A). To examine the expression of *CYP9Q3* at higher resolution, we used *in situ* hybridization with digoxigenin-labeled RNA probes to localize *CYP9Q3* expression in the brain and Malpighian tubules. This revealed that *CYP9Q3* is expressed at particularly high levels in the proximal regions of Malpighian tubules and where they join the midgut-hindgut junction and in several structures of the honeybee brain, including the optic and antennal lobes and the mushroom bodies (Figures 4B and 4C). Malpighian tubules are the functional insect equivalents of vertebrate kidneys, and these

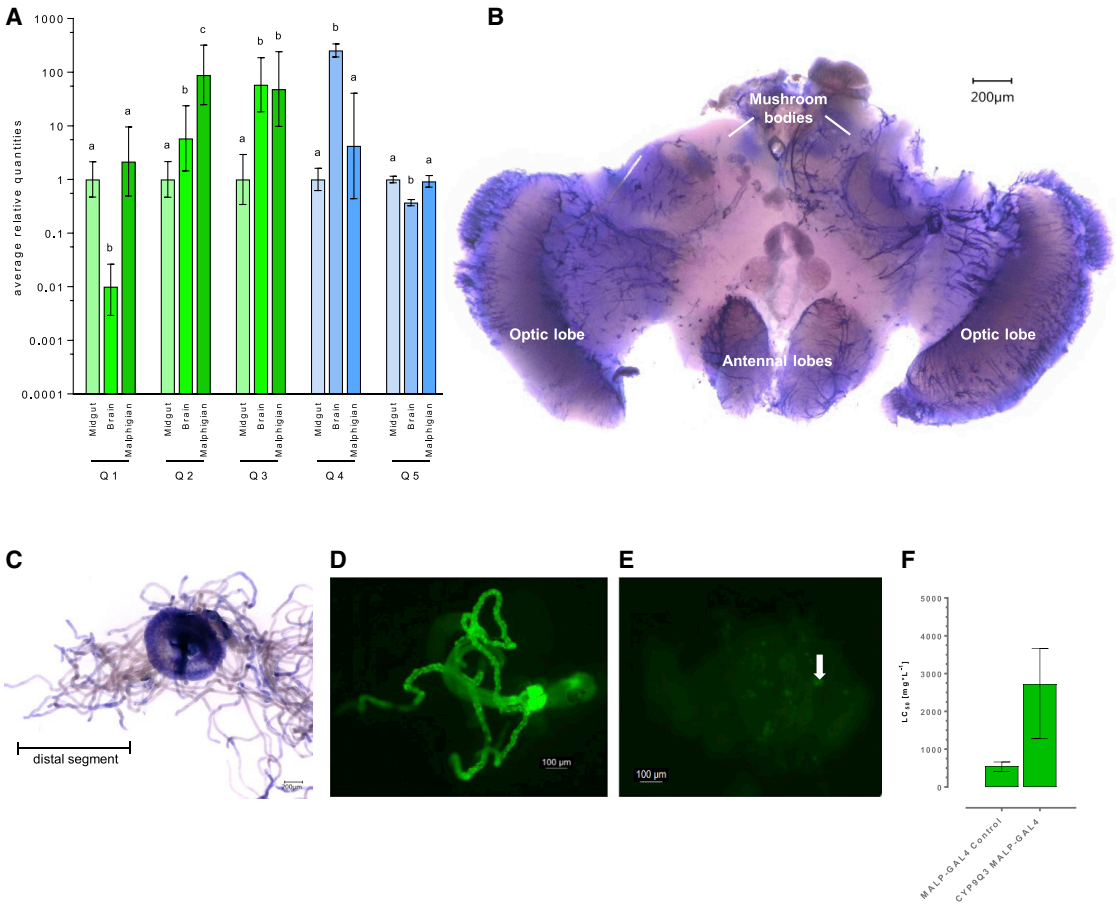

Figure 4. Tissue-Specific Expression and Functional Characterization of Honeybee and Bumble Bee Neonicotinoid-Metabolizing P450s

(A) Relative expression (fold change) of *A. mellifera* and *B. terrestris* thiacloprid metabolising CYP9Q genes in different tissues of worker bees measured by qPCR. Significant differences ($p < 0.01$) in expression between tissues is denoted using letters above bars as determined by One-Way ANOVA with post hoc testing (Benjamini and Hochberg).

(B and C) (B) Whole-mount *in situ* hybridization showing the distribution and abundance of the *AmCYP9Q3* transcript in the brain of a worker bee in different neuronal cells and in (C) the Malpighian tubules and distal midgut.

(D and E) Expression of green fluorescent protein in the Malpighian tubules and specific neurons of the *Drosophila* brain driven by the Malp-tub GAL4 line.

(F) Sensitivity of transgenic *Drosophila* to thiacloprid when the Malp-tub GAL4 line is used to drive expression of *AmCYP9Q3.* Error bars display 95% CLs.

osmoregulatory and detoxifying organs absorb solutes, water, and wastes from the surrounding haemolymph. The high expression of *CYP9Q3* in this tissue is therefore highly consistent with a primary role in xenobiotic detoxification. The expression of *CYP9Q3* and especially *CYP9Q4* in the bee brain suggests a secondary or additional site of detoxification against xenobiotics that cross the blood-brain barrier, and it is notable that the structures of the brain expressing *CYP9Q3* have been previously highlighted as sites of AChE activity and nAChR-like immunoreactivity [20]. Based on this finding, we explored the effect of specifically expressing *CYP9Q3* in the Malpighian tubules and the insect brain on sensitivity to thiacloprid by exploiting the GAL4/UAS system of *Drosophila*. Significant levels of thiacloprid resistance were observed in transgenic *Drosophila* when expression of *CYP9Q3* was directed to the Malpighian tubules and neuronal cells (ellipsoid body, pars intercerebralis, fan-shaped and large-field neurons) (Figures 4D and 4E and 4F), demonstrating that expression of *CYP9Q3* at these sites is sufficient to provide protection against this insecticide. Previous studies have examined the expression of honeybee *CYP9Q* P450s in different life stages of bees. For example, a recent study performed RNA-seq of different tissues in honeybee foragers, older workers which gather and process food, and nurses, young workers that care for brood [19]. While no change was observed in *CYP9Q3* expression in the Malpighian tubules and midgut between the two worker roles, foragers showed higher levels of expression in the mandibular and hypopharyngeal glands [19]. These findings were consistent with a second study, which examined the expression of *CYP9Q1–3* in the legs and antennae of newly eclosed workers, nurses, and foragers and observed a pattern of increased expression with age [21]. The greater expression of these P450s in foragers is consistent with their increased exposure to xenobiotics compared to nurses, and their elevated expression in tissues that mediate nectar processing suggests that they may provide a first line of defense against dietary xenobiotics.

Sequencing of the honeybee genome and the discovery that it contains a reduced number of genes encoding detoxification

enzymes (including P450s) led to the suggestion that bees may be particularly sensitive to xenobiotics, including pesticides [22]. However, a subsequent meta-analysis of available toxicological data revealed that honeybees are, in fact, no more sensitive to insecticides than other insect species [23]. Both honeybees and bumble bees have undergone millions of years of selection to evolve mechanisms to overcome the diverse array of toxic compounds that occur naturally in their environment [6]. Although this does not include the relatively recently introduced synthetic insecticides, our study, in combination with previous work [15], demonstrates that these existing detoxification pathways can be recruited to protect bees from pesticides if sufficient similarity exists between their native substrate(s) and the synthetic compound in question. In this regard, although the diversity of native substrates that the CYP9Q subfamily can metabolize is not fully understood, all members of this subfamily in honeybees have been shown to metabolize the plant secondary metabolite quercetin with high efficiency, a flavonoid that is present in pollen and nectar, which inhibits mitochondrial ATP synthase [15].

In conclusion, these data demonstrate that the CYP9Q family of both honeybees and bumble bees contains critically important enzymes that define their sensitivity to neonicotinoids. This finding illustrates the importance of considering bee xenobiotic biotransformation pathways to predict, and potentially influence, the pharmacological and toxicological outcomes of insecticide use. For example, the knowledge and tools developed in this study can be harnessed to avoid negative pesticide-pesticide interactions [24] due to inhibition of these key defense systems. Furthermore, our findings, and those of previous studies that have uncovered the molecular and biochemical basis of pesticide selectivity [15, 25–29], can facilitate the development of compounds that show high efficacy against crop pests but low toxicity to nontarget beneficial insects. In this regard, the recombinant enzymes and transgenic *Drosophila* lines developed in our study can be used as screening tools to assess the metabolic liability of future insecticidal lead compounds and so ensure that they are rapidly broken down by these major xenobiotic detoxifying enzymes.

STAR★METHODS

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- METHOD DETAILS
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  - Receptor binding studies
  - Functional expression of bee P450s
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SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and three tables and can be found with this article online at https://doi.org/10.1016/j.cub.2018.02.045.

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AUTHOR CONTRIBUTIONS

C.M., B.J.T., M.Z., K.B., E.R., G.H., K.S.S., C.T.Z., R.A.H., B.L., R.R., L.K., M.K., C.B., and R.N. performed experiments and analysis. C.B. and R.N. conceived and designed the study and drafted the manuscript. C.B., R.N., J.B., M.S.W., T.G.E.D., and L.M.F. directed the study. All authors helped write the manuscript.

DECLARATION OF INTERESTS

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alkaline phosphatase labeled antidigoxygenin antibody	abcam	Cat# ab6212
Biological Samples		
Bumblebee Colony	Agralan UK Ltd	Cat# M644
Chemicals, Peptides, and Recombinant Proteins		
NutriFly premix food	SLS	Cat# FLY1034
Phusion HF DNA polymerase	Thermo Fisher	Cat# 10024537
SYBR Green JumpStart Taq Readymix	Sigma-Aldrich	Cat# S4438500RXN
Bradford reagent	Sigma-Aldrich	Cat# B6916-500ML
NADPH	Sigma-Aldrich	Cat# N1630-25MG
Glutathione oxidized	Sigma-Aldrich	Cat# 64501
Glutathione reductase	Sigma-Aldrich	Cat# G3664
7-Hydroxycoumarin (HC)	Sigma-Aldrich	Cat# 202-240-3
7-Hydroxy-4-(trifluoromethyl)coumarin (HFC)	Sigma-Aldrich	Cat# 368512-250MG
7-methoxy-coumarin (MC)	Sigma-Aldrich	Cat# W515809-25G
7-Methoxy-4-(tri-fluoromethyl)-coumarin (MFC)	Sigma-Aldrich	Cat# T3165-100MG
7-ethoxy-coumarin (EC)	Sigma-Aldrich	Cat# E1379-100MG
7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC)	Sigma-Aldrich	Cat# 5057-5MG
7-ethoxy-4-trifluoro-methylcoumarin (EFC)	Sigma-Aldrich	Cat# 46127-100MG
7-benzyloxymethoxy-4-trifluoromethyl coumarin (BOMFC)	Sigma-Aldrich	Cat# 5047-5MG
Bovine Serum Albumin (BSA)	Sigma-Aldrich	Cat# P0834-10X1ML
Piperonyl butoxide (PBO)	Sigma-Aldrich	Cat# 291102-100ML
Pollen	Sussex Wholefoods	Cat# 7BEP2
Critical Commercial Assays		
ISOLATE II RNA Mini Kit	Bioline	Cat# BIO-52073
SuperScript III Reverse Transcriptase kit	Invitrogen	Cat# 18080044
Imidacloprid	Bayer CropScience	n/a
Thiacloprid	Bayer CropScience	n/a
Acetamiprid	Bayer CropScience	n/a
Bac-to-Bac Baculovirus Expression System	GIBCO	Cat# 10359016
NADPH Regeneration system	Promega	Cat# V9510
SsoAdvanced Universal SYBR® Green Supermix	BIO-RAD	Cat# 1725271
PicoPure RNA Isolation Kit	Thermo Fisher	Cat# KIT0204
iScript cDNA Synthesis Kit	BIO-RAD	Cat# 1708891
Plant DNeasy Mini Kit	QIAGEN	Cat# 69104
Deposited Data		
See Table S3 for accession numbers of P450s characterized in this study	N/A	See Table S3
Experimental Models: Cell Lines		
Sf9	GIBCO	Cat# 11496015
High Five	GIBCO	Cat# B85502

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Drosophila melanogaster:13-20: "y^{1}w^{67c23}; P attP40 25C6," "1;2"	University of Cambridge	Stock 13-20
Drosophila melanogaster: Act5C-GAL4: ["y[1] w[*]; P(Act5C-GAL4-w)E1/CyO,""1;2"	Bloomington Stock Center	Cat# 25374
Drosophila melanogaster: *Malp-GAL4*: *w[*]; P{w[+mW.hs] = GawB}c42*	Bloomington Stock Center	Cat# 30835
Drosophila melanogaster: *UAS-GFP: w^{1118}; P{w^{+mC} = UAS-GFP.nls}14*	Bloomington Stock Center	Cat# 4775
Oligonucleotides		
See Supplemental Materials	N/A	See Table S2
Recombinant DNA		
Cytochrome P450 variants	GeneArt, CA, USA	See Table S3
Cytochrome P450 reductase (CPR)	GeneArt, CA, USA	GenBank: Q07994
pUASTattB40 Vector	Gift from Jacob Riveron, Liverpool School of Tropical Medicine	GenBank: EF362409.1
Gateway pDEST8 expression vector	Invitrogen	Cat# 11804010
Software and Algorithms		
Geneious v 9.1.8	Biomatters	https://www.geneious.com/download/
Genstat v 16	VSN International	https://www.vsni.co.uk/software/genstat/
SoftMax Pro 7	Molecular devices	https://www.moleculardevices.com/systems/microplate-readers/softmax-pro-7-software
GraphPad Prism v 7	GraphPad Software Inc.	https://www.graphpad.com/
SpectralWorks	SpectralWorks Ltd	https://www.spectralworks.com/
qbase^{+} v 3.1	Biogazelle	https://www.qbaseplus.com/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests may be directed to and will be fulfilled by the Lead Contact, Chris Bass (chris.bass@exeter.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Insects

Adult honeybees were obtained from open hives during the summer of 2014-2016 that were maintained pesticide-free by bee keepers at Bayer AG, CropScience Division, Monheim, Germany. Bumblebee colonies were purchased from Agralan UK Ltd and maintained in constant darkness at 25°C, 50% RH. The colonies were fed *ad libitum* on the nectar substitute, Biogluc®, and pollen was supplied to colonies every 2 days.

The *Drosophila melanogaster* stock 13-20 ["y^{1}w^{67c23}; P attP40 25C6," "1;2"] obtained from the University of Cambridge was used to create all transgenic lines. Virgin females of this line were crossed to males of the Act5C-GAL4 strain ["y[1] w[*]; P(Act5C-GAL4-w)E1/CyO,""1;2"] (Bloomington Stock Center) to activate transgene expression (see below for description of methods). The *Malp-GAL4* strain [*w[*]; P{w[+mW.hs] = GawB}c42*] (Bloomington Stock Center) which expresses *GAL4* in the Malpighian tubules and specific neuronal cells (ellipsoid body, pars intercerebralis, fan shaped and large field neurons), was used to drive the expression of *CYP9Q3* in these tissues. The *UAS-GFP* strain [*w^{1118}; P{w^{+mC} = UAS-GFP.nls}14*] (Bloomington Stock Center) was used to visualize the sites of expression driven by *Act5C-GAL4* and *Malp-GAL4* drivers. All flies were reared on NutriFly food (NLS) at 24°C. Only female flies 2-5 days post eclosion were used for insecticide bioassays.

Insect cell lines

The Sf9 and High Five insect cell lines (ovarian cells from *Spodoptera frugiperda* and *Trichoplusia ni* respectively) were maintained in suspension culture under serum-free conditions at 27°C containing 25 μg/ml^{-1} gentamycin in SF-900 II SFM (GIBCO) and Express Five SFM (GIBCO), respectively.

METHOD DETAILS

Insecticide bioassays of *A. mellifera* and *B. terrestris*

Acute contact insecticide assays were performed on female *A. mellifera* following standard methods OECD 2013 [30]. Bioassays of *B. terrestris* were based on the OECD guidelines developed for honeybees [30] but with bees assayed in individual Nicot cages. Bees were starved of sucrose solution for up to 2 hr to encourage feeding during the experiment. Individual *B. terrestris* were fed with 20 ul of insecticide-sucrose solution at concentrations of 0.01, 0.1, 1, 10 and 100 ppm for imidacloprid and 10, 50, 100, 500 and 1000 ppm for thiacloprid. Controls were fed a solution of sucrose containing a concentration of acetone matching that of the highest treatment concentration. After 4-6 hr the syringes were assessed to see if bees had consumed the insecticide-sucrose solution. Those that had not consumed all of the solution were excluded from the experiment. Mortality was assessed 48 hr after feeding and lethal concentrations (LC_{50} values) were calculated by probit analysis using Genstat version 16 (VSN International). For synergist bioassays, *B. terrestris* or *A. mellifera* workers were first treated with 20 μg of piperonyl butoxide or 1 μg of aminobenzotriazole applied to the dorsal thorax. Synergist bioassays included an additional control group treated only with the synergist. 1 hr after synergist application, bees were then treated with the appropriate insecticide dosage as above.

Receptor binding studies

[^{3}H]imidacloprid (specific activity 1.406 GBq μmol^{-1}) displacement studies were conducted using membrane preparations isolated from frozen (−80°C) honeybee and bumble bee heads, respectively, following previously published protocols [9]. Briefly, bee heads weighing 10cg were homogenized in 200cml ice-cold 0.1cM potassium phosphate buffer, pH 7.4 containing 95cmM sucrose using a motor-driven Ultra Turrax blender. The homogenate was then centrifuged for 10cmin at 1200cg and the resulting supernatant filtered through five layers of cheesecloth with protein concentration determined using Bradford reagent (Sigma) and bovine serum albumin (BSA) as a reference. Assays were performed in a 96-well microtiter plate with bonded GF/C filter membrane (Packard UniFilter-96, GF/C) and consisted of 200 μL of homogenate (0.48cmg protein), 25 μL of [^{3}H]imidacloprid (576cpM) and 25 μL of competing ligand. Ligand concentrations used ranged from 0.001 to 10c000cnM and were tested at least in duplicate per competition assay. The assay was started by the addition of homogenate and incubated for 60cmin at room temperature. Bound [^{3}H]imidacloprid was quantified by filtration into a second 96-well filter plate (conditioned with ice-cold 100cmM potassium phosphate buffer, pH 7.4 (including BSA 5cg liter−1)) using a commercial cell harvester (Brandel). After three washing steps (1cml 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 10cμM unlabelled imidacloprid. All binding experiments were repeated twice using three replicates per tested ligand concentration. Data were analyzed using a 4 parameter logistic non-linear fitting routine (GraphPad Prism version 7 (GraphPad Software, CA, USA)) in order to calculate I_{50}-values (concentration of unlabelled ligand displacing 50% of [^{3}H]imidacloprid from its binding site).

Functional expression of bee P450s

All bee P450 (see Table S3 for accession numbers) and house fly NADPH-dependent cytochrome P450 reductase (CPR) (GenBank accession number Q07994) genes were obtained by gene synthesis (Geneart, CA, USA) and inserted into the pDEST8 expression vector (Invitrogen). Codon optimization of all bee genes was used to optimize expression in lepidopteran cell lines. The PFastbac1 vector with no inserted DNA was used to produce a control virus. The recombinant baculovirus DNA was constructed and transfected into *Trichoplusia ni* (High five cells, Thermo Fisher) using the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer's instructions. The titer of the recombinant virus was determined following protocols of the supplier. High Five cells grown to a density of 2×10^6 cells ml^{-1} were co-infected with recombinant baculoviruses containing each bee P450 and CPR with a range of MOI (multiplicity of infection) ratios to identify the optimal conditions. Control cells were co-infected with the baculovirus containing vector with no insert (ctrl-virus) and the recombinant baculovirus expressing CPR using the same MOI ratios. Ferric citrate and δ-aminolevulinic acid hydrochloride were added to a final concentration of 0.1 mM at the time of infection and 24 h after infection to compensate the low levels of endogenous heme in the insect cells. After 48 h, cells were harvested, washed with PBS, and microsomes of the membrane fraction prepared according to standard procedures and stored at −80°C [31]. Briefly, pellets were homogenized for 30 s in 0.1M Na/K-phosphate buffer, pH 7.4 containing 1mM EDTA and DTT and 200mM sucrose using a Fastprep (MP Biomedicals), filtered through miracloth and centrifuging for 10 min at 680 g at 4°C. The supernatant was then centrifuged for 1 h at 100,000 g at 4°C, with the pellet subsequently resuspended in 0.1M Na/K-phosphate buffer, pH 7.6 containing 1mM EDTA and DTT and 10% glycerol using a Dounce tissue grinder. P450 expression and functionality was estimated by measuring CO-difference spectra in reduced samples using a Specord 200 Plus Spectrophotometer (Analytik Jena) and scanning from 500 nm to 400 nm [31]. The protein content of samples was determined using Bradford reagent (Sigma) and bovine serum albumin (BSA) as a reference.

Metabolism assays and UPLC-MS/MS analysis

Metabolism of thiacloprid, imidacloprid and acetamiprid were assayed by incubating each recombinant bee P450/CPR (50-80μg of protein/assay) or ctrl-virus/CPR microsomes in 0.1 M potassium phosphate buffer with an NADPH-regenerating system (Promega; 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, 0.4 U mL^{-1} glucose-6- phosphate dehydrogenase) and substrate (10-25 μM; 0.78125 – 200 μM for enzyme kinetics) at 30°C for 2 h (*A. mellifera* P450s all insecticides), or 27°C for 45 min (*B. terrestris* P450s for imidacloprid and thiacloprid) or 60 min (*B. terrestris* P450s for acetamiprid). The total assay volume was

200 μL using three replicates for each data point. Microsomes incubated without NADPH served as a control. The assay was stopped by the addition of ice-cold acetonitrile (to 80% final concentration), centrifuged for 10 min at 3000 g and the supernatant subsequently analyzed by tandem mass spectrometry as described previously [18]. For the chromatography on a Waters Acquity HSS T3 column (2.1x50mm, 1.8μm), acetonitrile/water/0.1% formic acid was used as the eluent in gradient mode. For detection and quantification in positive ion mode, the MRM transitions 253 > 186, 269 > 202 (thiacloprid, OH-thiacloprid), 256 > 175, 272 > 191 (imidacloprid, OH-imidacloprid) and 223 > 126, 209 > 126 (acetamiprid and N-desmethyl acetamiprid) were monitored. The peak integrals were calibrated externally against a standard calibration curve. The linear range for the quantification of neonicotinoid insecticides and their hydroxylated (thiacloprid and imidacloprid) and N-desmethylated (acetamiprid) metabolites was 0.1 to 1000 ng mL^{-1}. Recovery rates of parent compounds using microsomal fractions without NADPH were normally close to 100%. Substrate turnover from two independent reactions were plotted versus controls and Michaelis-Menten kinetics determined using GraphPad Prism version 7 (GraphPad Software, CA, USA).

Functional activity of recombinant P450s against fluorescent model substrates

The activity of individual *A. mellifera* and *B. terrestris* recombinant P450s were tested against seven fluorescent model substrates (all purchased from Sigma); 7-methoxy-coumarin (MC), 7-Methoxy-4-(tri-fluoromethyl)-coumarin (MFC), 7-ethoxy-coumarin (EC), 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC), 7-ethoxy-4-trifluoro-methylcoumarin (EFC), 7-benzyloxymethoxy-4-trifluoromethyl coumarin (BOMFC), and 7-p-methoxy-benzyloxy-4-trifluoro coumarin (MOBFC). Assays were carried out in black flat-bottomed 96-well plates in a 100 μL reaction containing 2 pmol of P450 per 50 μg of protein (*B. terrestris*) or 50 μg/well (*A. mellifera*), 1 mM of NADPH (Sigma) and 50 mM of a model substrate (Sigma). Three replicates were carried out for each data point. P450s incubated without NADPH and wells containing only potassium phosphate buffer served as controls. Samples were incubated at 25°C for 45 min (*B. terrestris*) or 30°C for 30 min (*A. mellifera*). Data were recorded using a SpectraMax Gemini XPS (*B. terrestris*) or a SpectraMax M2 (*A. mellifera*) at the excitation/emission wavelength suitable for each model substrate (MC, EC at 390-465, BFC, MFC at 410-535, EFC at 410-510 and BOMFC and MOBFC at 405-510 nm). As these substrates have a similar emission wavelength to NADPH (460 nm) the reaction was terminated prior to measurement by the addition of 100μL of a stop solution (25% DMSO, 0.05 M Tris/HCL pH10, 5 mM glutathione oxidised, and 0.2 U glutathione reductase). The reactions were incubated at 25°C (*B. terrestris*) or 30°C (*A. mellifera*) for a further 15 min and the data were recorded at the required excitation/emission wavelengths stated above. 7-hydroxy-4-(trifluoromethyl)-coumarin (HFC) (Sigma) was used to generate a standard curve for model substrates BFC, EFC, MFC, MOBFC, and BOMFC and 7-hydroxycoumarin (HC) (Sigma) for model substrates EC and MC. Each compound was diluted to a range of concentrations (0, 5, 10, 15, 20, 30, 50, 60, 80 and 100 pmol) using potassium phosphate buffer. 100 μL of each concentration was added to each well with four replicates for each data point. 100 μL of stop solution was then added and the contents mixed. The florescence was measured as above at the corresponding wavelengths for each model substrate. Microsoft Excel was used to calculate the y intercept for each compound. This was then subtracted from the average fluorescence measurement of each P450 along with the average control measurements.

Transgenic expression of bee P450s in *D. melanogaster*

A. mellifera (*AmCYP9Q1–3*) and *B. terrestris* (*BtCYP9Q4–5*) genes were codon optimized for *D. melanogaster* expression and cloned into the *pUASTattB* plasmid (GenBank: EF362409.1). *pUASTattB-CYP9Q1–3* and *pUASTattB-CYP9Q4–5* constructs were injected into preblastodermal embryos of a *D. melanogaster* strain carrying an *attP* docking site on chromosome 2 (*attP40*) and the *phiC31* integrase gene under the control of the vasa regulatory region on the X chromosome [*y w M(eGFP, vas-int, dmRFP)ZH-2A; P{CaryP} attP40*] [32]. The presence of the transgene was confirmed by PCR and sequencing. Genomic DNA was extracted from pools of 10 flies for each line using the Plant DNeasy Mini kit (QIAGEN) following the manufacturers protocol. 20 ng of this DNA was used as template in PCR using Phusion DNA polymerase (Thermo) following the manufacturers protocol and the primers listed in Table S2. Thermocycling conditions consisted of an initial denaturation step at 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 55°C for 20 s, 72°C for 1 min, and a final extension at 72°C for 5 min. Products were direct Sanger sequenced using the primers detailed in Table S2. Fly lines were made homozygous for the transgene integrations. The GAL4/UAS system was used to control the expression of bee CYP9Q genes in transgenic *D. melanogaster*. The strain *Act5C-GAL4* [y^1 *w*; P{Act5C-GAL4-w}E1/CyO*] was used to drive the expression of *CYP9Q1–3* and *CYP9Q4–5* genes ubiquitously under the control of the Actin5C regulatory sequence. The *Malp-GAL4* strain [*w[*]; P{w[+mW.hs] = GawB}c42*], which expresses *GAL4* in the Malpighian tubules and specific neuronal cells (ellipsoid body, pars intercerebralis, fan shaped and large field neurons), was used to drive the expression of *CYP9Q3* in these tissues. The *UAS-GFP* strain [w^{1118}*; P{*w^{+mC} *= UAS-GFP.nls}14*] was used to visualize the sites of expression driven by *Act5C-GAL4* and *Malp-GAL4* drivers. Transgene expression was confirmed by qPCR as previously described [33]. Total RNA was extracted from 4 pools of 10 adult flies of each line using the ISOLATE II RNA Mini Kit (Bioline) and reverse transcribed to cDNA using Superscript III reverse transcriptase (Invitrogen) following manufacturer protocols in both cases. PCR reactions (20 μL) contained 10 ng of cDNA, 10 μL of SYBR Green JumpStart Taq Readymix (Sigma), and 0.25 μm of each primer. Samples were run on a Rotor-Gene 6000 (Corbett Research) using temperature cycling conditions of: 2 min at 95°C followed by 40 cycles of 95°C for 15 s, 57°C for 15 s and 72°C for 20 s. Data were analyzed in Microsoft Excel according to the $\Delta\Delta C_T$ method [34] using the *RPL11* reference gene for normalization [33]. Full dose response bioassays were performed by feeding adult female flies a range of insecticide concentrations dissolved in sugar/agar. At least three replicates of 20 flies were carried out for each concentration. LC_{50} values were calculated as above.

Expression profiling of bee P450s

Bees were dissected and total RNA was prepared from tissues of single female bees using the PicoPure RNA Isolation Kit (ThermoFisher) as described by the manufacturer. 0.5 μg were used for cDNA synthesis using iScript (Biorad) according to the manufacturer's instructions. PCR reactions (10 μL) contained 2.5 μL of cDNA (7.8 ng), 5 μL of SsoAdvanced Universal SYBR Green Supermix (BioRad), and 0.25 μM of each primer (Table S2). Samples were run on a CFX384 Real Time System (BioRad) using the temperature cycling conditions of: 3 min at 95°C followed by 39 cycles of 95°C for 15 s, 64°C for 15 s and 60°C for 15 s. A final melt-curve step was included post-PCR (ramping from 65-95°C by 0.5°C every 5 s) to confirm the absence of any non-specific amplification. The efficiency of PCR for each primer pair was assessed using a serial dilution of 25 ng to 0.04 ng of cDNA. Each qPCR experiment consisted of at least 7 independent biological replicates with three technical replicates for each. Data were analyzed according to the $\Delta\Delta C_T$ method [34] using qbase+ Version: 3.1 (Biogazelle). The expression level was normalized to two validated reference genes [35–37] for each species. *Rpl32* (ribosomal protein L32), *GADPH* (glyceraldehyde 3-phosphate dehydrogenase), PAL2 (phospholipase A2) and *EEF1A* (elongation factor 1-alpha) of the honeybee and bumble bee respectively (Table S2). *In situ* hybridization with antibody labeled RNA probes was used to visualize the expression of *CYP9Q3* in the brain and Malpighian tubules of honeybees. Fragments of ~700 bp were amplified from honeybee cDNA by PCR using gene-specific primers (Table S2) containing the T7 promoter sequence at the end and served as templates for synthesis using the T7 RNA polymerase and digoxigenin-labeled ribonucleotides. Digoxigenin-labeled riboprobes were purified and hydrolyzed into 100-400 bp fragments with 0.1 M sodium carbonate. Tissues from cold-anaesthetized bees were then dissected in PBS, fixed overnight in 4% paraformaldehyde and dehydrated in a methanol series. Before hybridization tissues were rehydrated in PBS/0.1% Tween, pre-incubated overnight at 55°C in hybridization buffer (50% formamide, 5xSSC, 0.1% Tween, 100 μg ml^{-1} yeast tRNA, 200 μg ml^{-1} salmon sperm, 50 μg ml^{-1} heparin) and then hybridized with the diluted riboprobes (1.0-4.0 ug ml^{-1} in hybridization buffer) at 55°C. After extensive post-hybridization stringency washes samples were pre-blocked in 1% BSA for at least 1 h prior to overnight incubation with the pre-adsorbed alkaline phosphatase labeled antidigoxygenin antibody (1:2000 dilution in PBS/1% BSA/0.1% Tween). The signal was visualized with NBT/BCIP alkaline phosphatase substrates according to the manufacturer's instructions.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed in GraphPad Prism 7 (GraphPad Software) apart from qPCR analsyses, which were performed in qbase+ Version 3.1 (Biogazelle). Significant differences in expression in all qPCR experiments were determined using one-way ANOVA with post hoc testing (Benjamini and Hochberg). Significant differences in activity of recombinant P450s against thiacloprid and imidacloprid was determined using a Welch's t test. Statistical details of experiments (value of n, precision measures and definitions of significance) are provided in figure legends.

DATA AND SOFTWARE AVAILABILITY

The sequences reported in this paper are all available in online sequence repositories (see Table S3).

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Supplemental Information

Unravelling the Molecular Determinants of Bee Sensitivity to Neonicotinoid Insecticides

Cristina Manjon, Bartlomiej J. Troczka, Marion Zaworra, Katherine Beadle, Emma Randall, Gillian Hertlein, Kumar Saurabh Singh, Christoph T. Zimmer, Rafael A. Homem, Bettina Lueke, Rebecca Reid, Laura Kor, Maxie Kohler, Jürgen Benting, Martin S. Williamson, T.G. Emyr Davies, Linda M. Field, Chris Bass, and Ralf Nauen

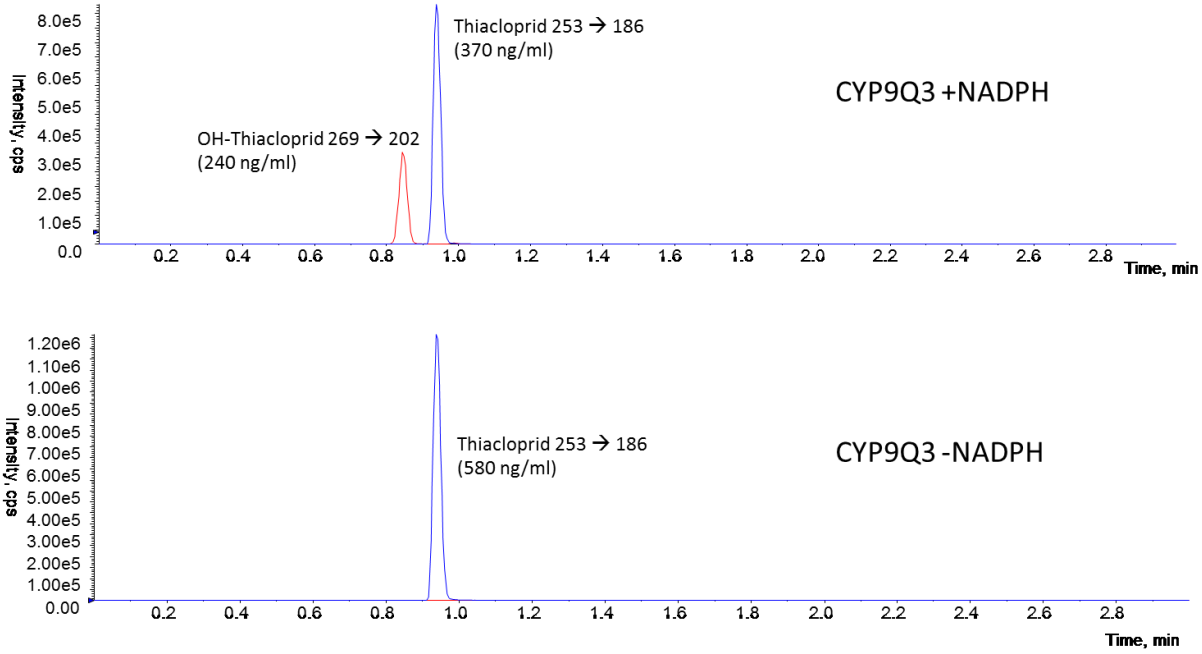
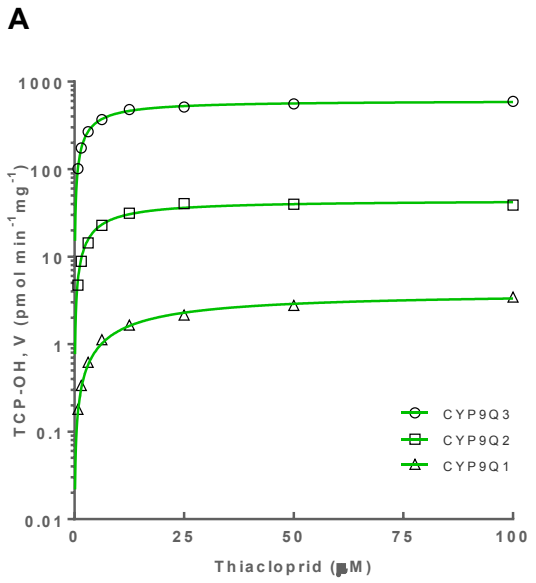
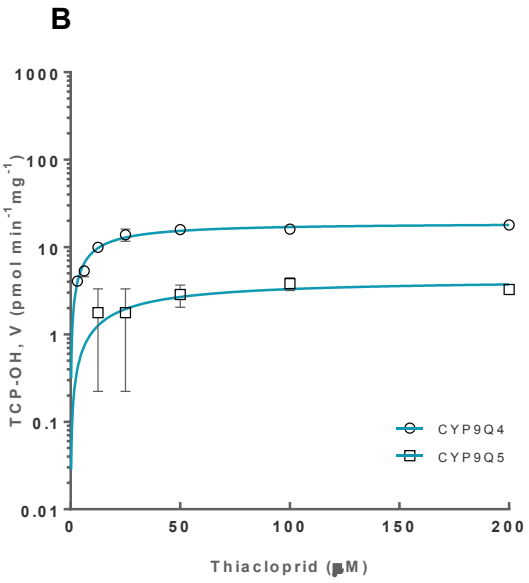


Figure S1. LC-MS/MS analysis of thiacloprid metabolism by CYP9Q3. Related to Figure 2. LC-MS analysis of thiacloprid metabolism. Typical MRM chromatograms of the CYP9Q3 catalysed formation of OH-thiacloprid with and without NADPH. Ion transition of thiacloprid $[M+H]^+$ 253 and OH-thiacloprid $[M+H]^+$ 269 to their fragments m/z 186 and m/z 202 are measured, respectively.





	CYP9Q1	CYP9Q2	CYP9Q3
V_{max}	3,92 ±0,08	44,31±1,01	607,8±5,48
K_m	17,83±1,08	5,65±0,49	3,92±0,14

	CYP9Q4	CYP9Q5
V_{max}	18,94±0,64	4,29±0,71
K_m	11,82±1,51	30,04±14,85

Figure S2. Michaelis-Menten kinetics of thiacloprid hydroxylation by *A. mellifera* and *B. terrestris* metabolising P450s analysed by non-linear regression. Related to Figure 2. A, B, Michaelis-Menten kinetics plots of thiacloprid hydroxylation catalyzed by AmCYP9Q1-3 **(A)** and BtCYP9Q4-5 **(B)**. The apparent K_m and V_{max} values for thiacloprid are indicated below the respective graphs. Data points are mean values ± SD (n=3).

	Bt CYP9P1	Bt CYP9P2	Bt CYP9R1	Bt CYP9Q4	Bt CYP9Q5	Am CYP9Q1	Am CYP9Q3	Am CYP9Q2
Bt CYP9P1		63.953%	41.454%	37.795%	37.795%	37.305%	36.471%	38.235%
Bt CYP9P2	63.953%		41.765%	40.354%	40.748%	37.305%	37.451%	39.412%
Bt CYP9R1	41.454%	41.765%		42.185%	42.938%	38.716%	41.892%	42.402%
Bt CYP9Q4	37.795%	40.354%	42.185%		93.143%	55.340%	56.840%	61.163%
Bt CYP9Q5	37.795%	40.748%	42.938%	93.143%		54.563%	57.225%	61.726%
Am CYP9Q1	37.305%	37.305%	38.716%	55.340%	54.563%		53.488%	56.589%
Am CYP9Q3	36.471%	37.451%	41.892%	56.840%	57.225%	53.488%		57.500%
Am CYP9Q2	38.235%	39.412%	42.402%	61.163%	61.726%	56.589%	57.500%	

Figure S3. Heat map showing the levels of sequence identity between *A. mellifera CYP9Q1-3* and *B. terrestris CYP9* genes. Related to Figure 2.

Application	Insecticide	Synergist	LD_{50} (µg/bee)	95% CI	Slope	± SE	Synergism Ratio
Topical	Imidacloprid	None	0.38	0.12 - 1.45	0.6	0.11	n/a
	Thiacloprid	None	>100	n/a	n/a	n/a	n/a
Oral	Imidacloprid	None	0.038	0.012 - 0.075	1.5	0.44	n/a
		PBO	0.032	0.016 - 0.05	1.9	0.41	1.2
	Thiacloprid	None	19.68	13.45 - 26.88	1.8	0.26	n/a
		PBO	4.73	2.55 - 7.71	1.4	0.24	4.2

Table S1. Sensitivity of *Bombus terrestris* to imidacloprid and thiacloprid in insecticide bioassays. Related to Figure 1. Neonicotinoid acute contact and acute oral LD_{50} values (±95% confidence intervals) and slope (±SE) for *Bombus terrestris* 48 hours after application of insecticide. Synergism ratio is also shown, where the P450 inhibitor piperonyl butoxide (PBO) was used.

Primers	**Sequence**	**Use**
AmCyp9Q3 F1	5'-GATGTGCGTCGAGAGTTTCC-3'	qPCR (CYP9Q3)
AmCyp9Q3 R1	5'-CTGTCCGGGTCGAATTTGTC-3'	qPCR (CYP9Q3)
AmCyp9Q2 F1	5'-ATGGAAGGAGCACAGGAACA-3'	qPCR (CYP9Q2)
AmCyp9Q2 R1	5'-ACGTCGTTGGTGTATCTGGT-3'	qPCR (CYP9Q2)
AmCyp9Q1 F1	5'-GGAGGAGGGGAAGAGAGGTA -3'	qPCR (CYP9Q1)
AmCyp9Q1 R1	5'-CCTCCTGAAGCCTCTGTTGA-3'	qPCR (CYP9Q1)
AmRpl32 F1	5'-AGTAAATTAAAGAGAAACTGGCGTAA-3'	qPCR (reference gene)
AmRpl32 R1	5'-TAAAACTTCCAGTTCCTTGACATTAT-3'	qPCR (reference gene)
AmGADPH F1	5'-ACCTTCTGCAAAATTATGGCGA-3'	qPCR (reference gene)
AmGADPH R1	5'-CACCTTTGCCAAGTCTAACTGTTAAG-3'	qPCR (reference gene)
BtCyp9Q4 F1	5'-TATTCCACCAACGCCACTGT-3'	qPCR (CYP9Q4)
BtCyp9Q4 R1	5'-GGTCCACTTCCTTGTATGCG-3'	qPCR (CYP9Q4)
BtCyp9Q5 F1	5'-CCTACGATGCTCTAAGCGAGATG-3'	qPCR (CYP9Q5)
BtCyp9Q5 R1	5'-ATTCTCGTAATATTGAGGATCGCG-3'	qPCR (CYP9Q5)
BtPal F1	5'-TGTCGGTATCTACGCGCCTG-3'	qPCR (reference gene)
BtPal R1	5'-TTGGTGGATGCTTGTCAGTC-3'	qPCR (reference gene)
BtEEF1A F1	5'-AGAATGGACAAACCCGTGAG-3'	qPCR (reference gene)
BtEEF1A R1	5'-CACAAATGCTACCGCAACAG-3'	qPCR (reference gene)
D099 pUAST F	TCACTGGAACTAGGCTAGCA-3'	Sequence validation of transgenic flies
D102 pUAST F	5'-GGATCCAAGCTTGCATGCCTG-3'	sequence validation of transgenic flies
D100 pUAST R	5'-AAAGGCATTCCACCACTGCT-3'	sequence validation of transgenic flies
D101 pUAST R	5'-CCACCACTGCTCCCATTCAT-3'	sequence validation of transgenic flies
AmCyp9Q3 F3	5'-TGGAAGGAGCACAGGAACAT-3'	in situ hybridisation (CYP9Q3)
AmCyp9Q3 R6-T7	5'-TAATACGACTCACTATAGGGAGATGATCACGGCGTCCATGTAT-3'	In situ hybridisation (CYP9Q3)

Table S2. Sequence of oligonucleotide primers for, PCR, qRT-PCR and *in situ* hybridization used in this study. Related to STAR methods.

Species	Gene name	Accession Number
Bombus terrestris	CYP9Q4	XP_003393377
Bombus terrestris	CYP9Q5	XP_003393376.1
Bombus terrestris	CYP9P1	XP_020718545.1
Bombus terrestris	CYP9P2	XP_003393388.3
Bombus terrestris	CYP9R1	XP_003393379.1
Apis mellifera	CYP9Q1	XP_006562364
Apis mellifera	CYP9Q2	XP_392000
Apis mellifera	CYP9Q3	XP_006562363
Apis mellifera	CYP9R1	GB16803
Apis mellifera	CYP9S1	XP_016771487
Apis mellifera	CYP336A1	XP_001119981
Apis mellifera	CYP9P1	XP_006562365
Apis mellifera	CYP9P2	GB19055
Apis mellifera	CYP6AQ1	NP_001191991
Apis mellifera	CYP6AR1	XP_623362
Apis mellifera	CYP6AS1	GB16899
Apis mellifera	CYP6AS2	GB19197
Apis mellifera	CYP6AS3	GB15681
Apis mellifera	CYP6AS4	XP_395671
Apis mellifera	CYP6AS5	DQ232888
Apis mellifera	CYP6AS7	XP_006565064
Apis mellifera	CYP6AS8	XP_006565076
Apis mellifera	CYP6AS10	XP_016771320
Apis mellifera	CYP6AS11	XP_016771191
Apis mellifera	CYP6AS12	XP_397347
Apis mellifera	CYP6AS13	GB17831
Apis mellifera	CYP6AS15	XP_623595
Apis mellifera	CYP6AS17	XP_006565063
Apis mellifera	CYP6AS18	XP_006565063
Apis mellifera	CYP6BC1	XP_016766476
Apis mellifera	CYP6BD1	XP_006564499
Apis mellifera	CYP6BE1	XP_624795

Table S3. Accession numbers of P450 sequences functionally expressed in this study. Related to STAR methods.

Chapter 4

Pharmacokinetics of three selected neonicotinoid insecticides upon contact exposure in the western honey bee, *Apis mellifera*

Authors: Marion Zaworra, Harald Koehler, Josef Schneider, Andreas Lagoja and Ralf Nauen

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Pharmacokinetics of Three Neonicotinoid Insecticides upon Contact Exposure in the Western Honey Bee, *Apis mellifera*

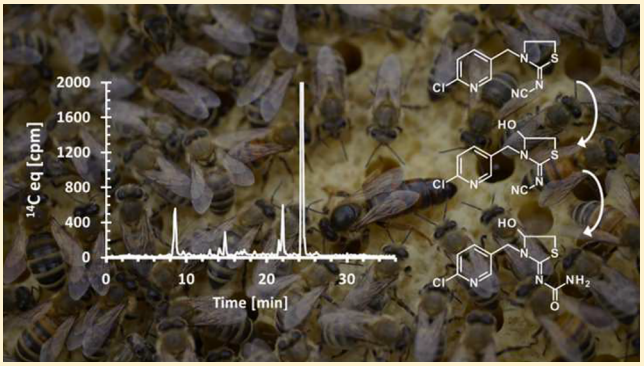
Marion Zaworra,[†,§] Harald Koehler,[†] Josef Schneider,[†] Andreas Lagojda,[†] and Ralf Nauen*[,†]

[†]Bayer AG, Crop Science Division, R&D, Alfred Nobel Str. 50, 40789 Monheim, Germany

[§]Institute of Crop Science and Resource Conservation, University of Bonn, 53115 Bonn, Germany

Supporting Information

ABSTRACT: Neonicotinoid insecticides differ in their acute contact toxicity to honey bees. We investigated the uptake, metabolic fate, and excretion of imidacloprid and two much less toxic chemotypes, thiacloprid and acetamiprid, upon contact exposure in honey bees because ADME data for this mode of entry are lacking. Pharmacokinetic parameters were analyzed by tracking a ^{14}C-label and by HPLC coupled to ESI-MS. Imidacloprid penetrates the honey bee cuticle much faster and more readily compared to thiacloprid and acetamiprid, thus revealing a pharmacokinetic component, i.e., faster penetration and higher steady-state internal body concentrations, contributing to its higher acute contact toxicity.

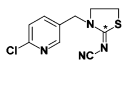
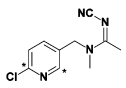
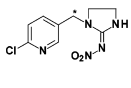


Neonicotinoid insecticides are a widely used class of chemistry with a number of commercially available compounds agonistically targeting the orthosteric binding site of insect nicotinic acetylcholine receptors (nAChR).[1,2] Because of their high efficacy, systemic properties, and low intrinsic toxicity toward vertebrates, neonicotinoids are used to control various sucking and chewing pest species. Their importance for pest control purposes is underlined by an insecticide market share of >20% in 2013.[3]

In the past years concerns have been raised about bee pollinator declines, and among various factors involved, insecticide use in crop protection is one factor under discussion.[4,5] Providing data on bee pollinator safety is a regulatory requirement for plant protection products. This is addressed in a tiered approach based on the initial acute oral and contact toxicity of compounds against western honey bees (*Apis mellifera*) according to OECD guidelines.[6] The acute contact toxicity of neonicotinoids on honey bees is well-characterized.[7] Interestingly, thiacloprid (TCP) and acetamiprid (ACT), both carrying a *N*-cyanoamidine pharmacophore, are almost 3 orders of magnitude less toxic when compared to *N*-nitroguanidine compounds such as imidacloprid (IMD), despite similar high-affinity binding to bee nAChR in vitro.[8] However, differences in physicochemical properties likely influence their cuticular penetration kinetics and thus acute toxicity upon contact application (Table 1).

Two recent studies tracked the in vivo metabolic fate of orally administered [^{14}C]-ACT[9] and [^{14}C]-IMD[10] (but not TCP) in honey bees, and the obtained metabolite profiles clearly indicated that the metabolism of both compounds is driven by cytochrome P450 monooxygenases (P450). This superfamily of membrane-bound hemoproteins is involved in the oxidative metabolism of many endogenous and exogenous compounds, including insecticides.[11]

Table 1. Acute Contact Toxicity to Honey Bees (24 h), nAChR Binding Affinity, and Physiochemical Properties of Thiacloprid, Acetamiprid, and Imidacloprid[a]

	Thiacloprid	Acetamiprid	Imidacloprid
Chemical Structure			
Pharmacophore	*N*-Cyano	*N*-Cyano	*N*-Nitro
Contact LD_{50} (μg a.i. bee^{-1})[7]	14.6	7.07	0.0179
IC_{50} (nM) honey bee nAChR[8]	0.75	2.1	2.9
Log P_{ow} (at 20°C/ 22°C[a])[2]	1.26	0.8	0.57[a]
Molecular weight (g mol^{-1})[2]	252.72	222.68	255.66
Water solubility (g L^{-1} at 20°C / 25°C[b])[2]	0.185	4.2[b]	0.61

[a]The ^{14}C-label position is indicated by an asterisk.

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It is yet unknown if despite the faster metabolization a pharmacokinetic component, such as slower penetration and lower steady-state internal body concentrations, adds to the much lower acute contact toxicity of TCP and ACT as compared to IMD.

The insect's (outer) epicuticle is a waxy lipophilic layer, whereas the (inner) procuticle is rather hydrophilic and made of an aqueous chitin-protein complex; therefore, uptake and cuticular penetration rate of an insecticide largely depend on its physicochemical properties and the solvent used to administer it.[13] To assess the pharmacokinetics of [^{14}C]-TCP and [^{14}C]-ACT, separate doses well below the acute contact LD_{50} (~1 μg a.i. honey bee^{-1}) were applied onto the dorsal thorax of groups of honey bees split into three batches containing five bees each, whereas [^{14}C]-IMD was applied at a dose of ~10 ng a.i. honey bee^{-1} (see Experimental Section in the Supporting Information). At different elapsed time intervals, uptake, internal concentration, and excretion of respective [^{14}C]-equivalents were assessed. The metabolite profiles were qualitatively analyzed in honey bee extracts 4 h (TCP) and 24 h (ACT and IMD) after application.

All bees treated with TCP and ACT survived the treatment and showed no symptoms of poisoning throughout the experiment, whereas bees treated with IMD showed symptoms of poisoning after 2 h, resulting in complete paralysis after 24 h. Thiacloprid was rather slowly taken up by the bee's cuticle. After 24 h, 62 ± 7.2% of the recovered radiolabel was still present in the external rinse (Figure 1), whereas 14.6 ± 1.8% of the applied [^{14}C]-TCP equivalents were extracted from the body (internal) and 23.4 ± 8.9% was excreted, thus indicating clearance (Figure 1). Honey bee extracts analyzed by HPLC ESI-MS revealed that TCP dominates, followed by hydroxylated TCP, the corresponding hydroxylated TCP-amide, an unknown metabolite, and traces of TCP-amide (Figure 1). Furthermore, 6-chloronicotinic acid (6-CNA) was detected by MS only, because it does not have a [^{14}C]-label (data not shown). Our previous molecular in vitro study on the role of 26 recombinantly expressed clade 3 P450s in TCP metabolism revealed CYP9Q3 as the key enzyme forming hydroxylated TCP.[12] The capacity to form this major metabolite was confirmed in the present study in vivo. None of the 26 honey bee P450's analyzed by Manjon et al. (2018)[12] catalyzed the conversion of the TCP *N*-cyano pharmacophore to its amide, identified in this study. However, it is generally considered to be a hydrolytic reaction process, though it has been recently demonstrated that the *N*-cyano group of pinacidil, an antihypertensive drug, is converted to the corresponding amide by human CYP3A4.[14]

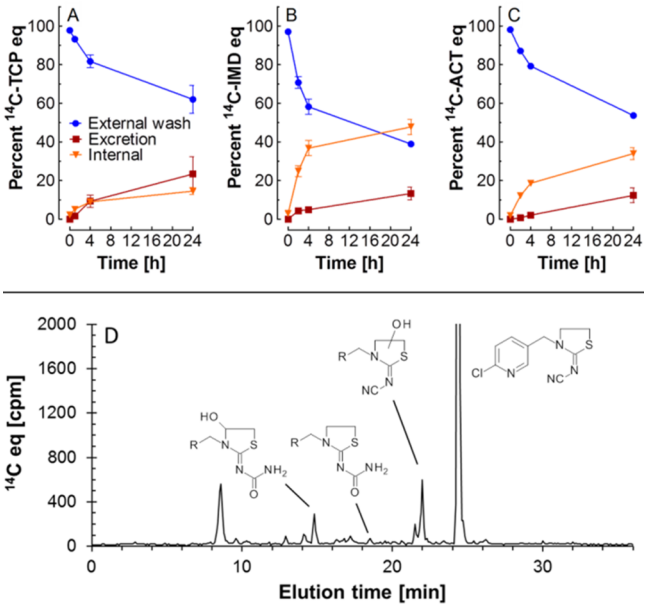

Figure 1. Distribution of [^{14}C]-equivalents expressed as percent recovered radioactivity at different times after topical application of honey bee adults with (A) [^{14}C]-TCP, (B) [^{14}C]-IMD, and (C) [^{14}C]-ACT. Data are mean values ± SEM (n = 3). (D) HPLC radiohistogram of a sample extracted from honey bees, treated with [^{14}C]-TCP 4 h prior to metabolite extraction for qualitative metabolite profiling using ESI-MS. Only those [^{14}C]-TCP metabolites clearly identified are shown (R refers to the 6-chloro-3-pyridyl group).

The uptake kinetics of [^{14}C]-ACT was comparable to that of [^{14}C]-TCP and not significantly different ($p > 0.05$) when comparing the amount of radiolabel in the external rinse at different elapsed time intervals (Figure 1). The total recovery of radiolabel washed off the cuticle after 24 h was 54 ± 0.76%, suggesting a better uptake when compared to TCP. In contrast to TCP, the internal amount of [^{14}C]-ACT equivalents was significantly higher (34 ± 3.1%, $p < 0.05$), suggesting a slower clearance of ACT and its corresponding metabolites by excretion. The main metabolite formed in honey bees 24 h after contact exposure was 6-CNA, followed by *N*-desmethyl-acetamiprid, ACT, and 6-chloro picolyl alcohol (6-CPA) (see Figure S1). The metabolic fate of acetamiprid upon topical application is in line with a previously published study tracking the fate of orally administered [^{14}C]-ACT.[9] The main metabolites identified 24 h after oral administration in different tissues such as rectum and intestine were 6-CNA, *N*-desmethyl ACT, and 6-CPA, results that are comparable to our study. The lower turnover rate of ACT compared to TCP in vivo could be explained by the lower metabolization capacity driven by CYP9Q-enzymes.[12] One of the key enzymes in ACT metabolism, catalyzing the *N*-desmethylation of ACT, was CYP9Q2; however, the turnover rate was much lower compared to CYP9Q3, facilitating the hydroxylation of TCP.[12]

The pharmacokinetics of [^{14}C]-IMD differs substantially from both TCP and ACT, because it penetrates the honey bee cuticle much faster and readily accumulates in the bee body (Figure 1), thus explaining the quick onset of symptoms of poisoning already 2 h after topical application. Four hours after application only 58 ± 3.9% of radiolabel could be recovered in the external rinse, whereas 37 ± 3.9% of [^{14}C]-IMD equivalents were detected in bee body extracts. This is in contrast to and significantly different ($p < 0.05$) than the 9.0 ± 0.69% and 18 ± 1.5% of internally cumulated [^{14}C]-TCP and [^{14}C]-ACT equivalents 4 h after application, respectively. After 24 h of contact exposure the internal amount of [^{14}C]-IMD equivalents reached 47.8 ± 4.0%, whereas excreted levels where rather low, with an average of 12% ± 3.8% (Figure 1). The largest proportion of radiolabel found in honey bee extracts 24 h after treatment was the parent compound IMD itself (see Figure S2). Additionally, we identified 6-CNA, mono- and dihydroxy IMD, and the olefin metabolite, which is known to be more toxic to honey bees than IMD, particularly because of its higher affinity to honey bee nAChR preparations.[8] The enrichment of [^{14}C]-IMD and some of its metabolites, particularly IMD-monohydroxy and olefin, within the bee body explains the prolonged paralysis of treated bees

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24 h after application. Our results confirm a recently published study investigating the pharmacokinetics and metabolic fate of IMD after oral application.[10] It has been demonstrated earlier that two of the detected IMD metabolites, monohydroxy-imidacloprid and the olefin, have an intrinsic toxicity comparable to that of the parent compound itself.[8] In conclusion, rapid penetration combined with the formation of two intrinsically active metabolites are factors contributing to the high acute toxicity of IMD in honey bees upon contact exposure. A recently conducted study on the temporal dynamics of whole body residues of IMD upon oral uptake of doses as high as 41 ng/bee indicated postnarcosis recovery due to IMD detoxification/clearance.[15] However, it is worth mentioning that there are different views concerning the issue of IMD persistence in bees.[16,17]

The penetration and uptake of neonicotinoid insecticides through honey bee cuticles has not been studied in detail yet, but they are likely to be influenced by neonicotinoid polarity, the chosen solvent, and the composition of the cuticle. In the past it has been shown that the rates of insecticide penetration are often inversely related to their partition coefficient.[13] The penetration of insecticides topically applied to the insect cuticle in acetone, as in the present study, is suggested to be driven by their polarity, because the waxy layer is neutralized, thus allowing polar compounds to cross the hydrophilic barrier of the procuticle as recently shown in cockroaches.[18] Our results reveal a similar trend by comparing the difference in polarity between IMD (logP 0.57) and TCP (logP 1.26), resulting in approximately 60% and 30% cuticular penetration, respectively.

To conclude, we have shown that a pharmacokinetic component contributes to the much lower honey bee acute contact toxicity of TCP and ACT compared to that of IMD. This work closes a knowledge gap related to the pharmacokinetics of neonicotinoids and complements our previous finding that P450s of the CYP9Q-subfamily are key molecular determinates for neonicotinoid selectivity in honey bees.[12]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.8b00315.

Additional figures and experimental procedures (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: ralf.nauen@bayer.com. Phone: +2173 384441.

ORCID

Ralf Nauen: 0000-0002-7525-8589

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The authors are all employees of Bayer AG.

Notes

The authors declare the following competing financial interest(s): Bayer AG is a manufacturer of certain neonicotinoid insecticides.

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■ ABBREVIATIONS

IC_{50}, inhibitor concentration reducing 50% binding of radioligand; LD_{50}, lethal dose resulting in 50% mortality; Log P_{ow}, partition coefficient n-octanol/water

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Supporting information

Pharmacokinetics of three neonicotinoid insecticides upon contact exposure in the western honey bee, *Apis mellifera*

Marion Zaworra[†,§], Harald Koehler[†], Josef Schneider[†], Andreas Lagojda[†] and Ralf Nauen[*,†]

[†]Bayer AG, Crop Science Division, R&D, Alfred Nobel Str. 50, 40789 Monheim, Germany

[§]Institute of Crop Science and Resource Conservation, University of Bonn, 53115 Bonn, Germany

* Corresponding Author: Ralf Nauen
Tel: +49 2173 384441
Email address: ralf.nauen@bayer.com

Address: Bayer AG
Crop Science Division, R&D
Pest Control, Building 6260
Alfred Nobel Str. 50
40789 Monheim
Germany

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Material and methods

Solvents and radiolabeled insecticides

Acetonitrile (gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur., CAS 75-05-08) was purchased from Merck (Darmstadt, Germany). [^{14}C]-thiacloprid (label position thiazolidine-2-[^{14}C], specific activity 4.12 MBq/mg), [^{14}C]-acetamiprid (label position pyridyl-[^{14}C], specific activity 4.1 MBq/mg) and [^{14}C]-imidacloprid (label position methylene-[^{14}C], specific activity 4.44 MBq/mg) were obtained from Bayer AG (Crop Science Division, Germany).

Insects

All adult worker honey bees (*Apis mellifera*, Hymenoptera: Apidae) used in the studies were of mixed age and collected from queen-right colonies maintained by bee keepers (Bayer AG, Crop Science Division, Monheim, Germany). The colonies appeared healthy and no disease control was performed. The colonies were not exposed to chemical treatments (for Varroa mite control) for at least four weeks before testing.

Pharmacokinetics of [^{14}C]-labelled neonicotinoids

The *in vivo* metabolic fate of thiacloprid, acetamiprid and imidacloprid was tracked by a [^{14}C]-label. Worker honey bees were collected from hives in summer 2014 and 2016, anaesthetized with carbon dioxide and randomly placed in three metal cages (L 8.5 x W 4.5 x H 6.5 cm) lined with a filter paper in groups of 5 bees. The bees were kept in the laboratory in the dark at room temperature for 24 h prior to insecticide treatment. Sucrose solution (500 g litre^{-1} 50% v/w, Apipuder, Suedzucker, Germany) was provided by a syringe all the time. The next day, the respective [^{14}C]-labelled compound (thiacloprid and acetamiprid: ~ 1 μg a.i. honey bee^{-1}; imidacloprid: ~ 0.01 μg a.i. honey bee^{-1}) was dissolved in 2 μl acetone and applied onto the dorsal thorax of bees anesthetized with carbon dioxide using a Hamilton syringe (Model 701N, Hamilton® Company). The pharmacokinetic parameters were assessed 0 h, 2 h, 4 h and 24 h after application in the three batches containing each five bees/time point. The radioactivity measurement in liquid samples was carried out by liquid scintillation counting (LSC) (Scintillator Quicksafe A, Zinsser Analytic, Germany, device Beckmann LS 6500, Beckmann Coulter, Germany). To evaluate the remaining radiolabel on the cuticle the treated bees were washed five times in 10 mL MeCN/H_2O (9/1; v/v). The radioactivity in the external wash solutions obtained after cuticle rinsing was determined by LSC as described above. The honey bees were flash frozen in liquid nitrogen and stored at -80 °C until homogenization. Each of the three batches per time point were separately processed and the bees extracted by mechanical homogenization followed by ultra-sonication with 5 mL acetonitrile for two times and one final extraction step with MeCN/H_2O (9/1; v/v) to extract possible polar metabolites. The solids were separated from the extracts by centrifugation after each extraction step. The radioactivity in the extracts was determined by LSC. The extracts were combined and subjected to liquid-liquid extraction using *n*-heptane to remove cuticle wax components from the solvents. After phase separation, the acetonitrile phase was concentrated using a Jouan RC1022 vacuum concentrator (Thermo Scientific, Waltham,

USA). The radioactivity remaining in the *n*-heptane phase was determined by LSC. The radioactivity in the remaining solid phase (bee tissue) was measured by combustion in an oxygen atmosphere using an oxidizer Ox 120c (Harvey Instruments Co., USA) with automated sample application (Zinsser Analytic, Germany) followed by LSC of the released $^{14}CO_2$ in an alkaline scintillation cocktail. The radioactivity measured in honey bee extracts and combusted samples of remaining bee fragments is referred to as the internal amount of compound at the respective time points. To analyze the compound equivalents in the honey bee excretions, the filter paper of each cage was cut into pieces and rinsed with acetonitrile in a beaker covered with parafilm while shaking for 1 h. Moreover, the whole cage was wiped out with filter paper pieces wetted with MeCN. The radioactivity in the rinse solutions and swipe samples was measured by LSC. The washed filter papers were dried and combusted (see above) and the results included in the calculation of the excreted amount. The application dose based on total recovery of radiolabel was calculated at 0.76 ± 0.15 μg bee^{-1}, 0.98 ± 0.08 μg bee^{-1} and 0.0082 ± 0.0013 μg bee^{-1} for [^{14}C]-TCP, [^{14}C]-ACT and [^{14}C]-IMD, respectively, directly after treatment (mean value ± SD, n=3 (5 bees each)).

High Performance Liquid Chromatography (HPLC) and LC-MS/MS analysis

The extracted residues in honey bee extracts, wash solutions and excretion solution were analyzed by HPLC. Liquid chromatography was performed on an 1100/1200 Series HPLC (Agilent, Waldbronn, Germany) equipped with a Nucleodur C18 Gravity column (4.0 x 250 mm, 5 µm particle size). The eluents used were 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). Gradient elution was conducted starting at 0% B for 5 min, increasing to 40% B within 25 min and 95% B within 30 min, maintained at 95% B for five minutes followed by re-equilibration at 0% B. The flow rate of 0.8 mL/min was directed to an Agilent 1260 Fraction Collector (Agilent, Waldbronn, Germany). Time based fractions of 6 sec were taken into a solid scintillation microtiter plate, LumaPlate 384, (Perkin Elmer, Waltham, MA, USA). The obtained plates were dried in a vacuum concentrator (Christ, Osterode, Germany). The measurement was carried out by a multichannel detector MicroBeta2 (Perkin Elmer, Waltham, MA, USA). The histograms were electronically and quantitatively analyzed by the software package Wallac TopCount Connector (BBS GmbH, Germany). The electro-spray ionisation MS spectra (ESI) were obtained with a Q Exactive mass spectrometer (Thermo, San Jose, CA, U.S.A.). The flow from the HPLC column was split between UV-detector and MS spectrometer.

Statistical analysis

To examine differences in the results obtained for TCP, ACT and IMD, analysis of variance (ANOVA), followed by Tukey´s multiple comparison test was carried out using GraphPad Prism 5 (GraphPad Software Inc., La Jolla CA, USA).

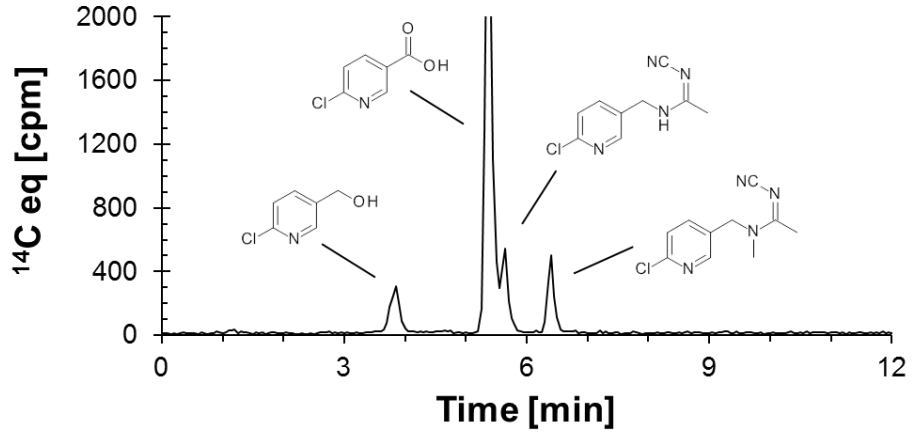

Figure S1. HPLC LC-MS/MS based analysis of radiolabeled metabolites extracted from adult honey bees 24 h upon application of [^{14}C]-acetamiprid.

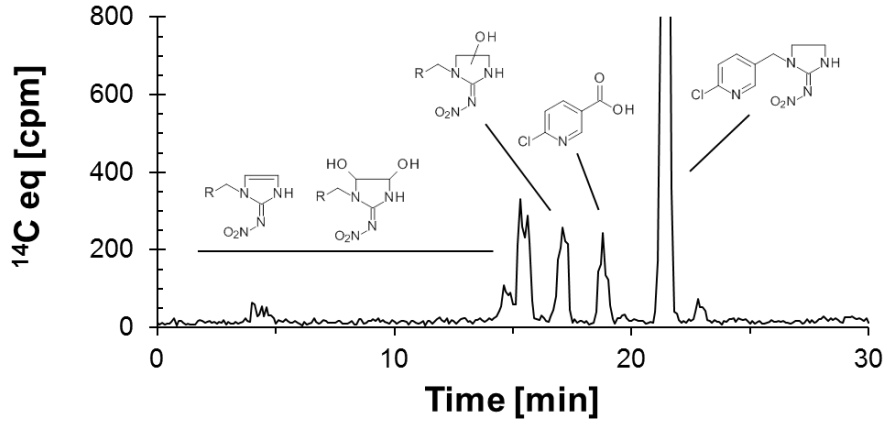

Figure S2. HPLC LC-MS/MS based analysis of radiolabeled metabolites extracted from adult honey bees 24 h upon application of [^{14}C]-imidacloprid.

Chapter 5 - Concluding Discussion

Over the last decade, there has been a controversial debate about the potential adverse impact of crop protection products on honey bee health culminating in speculations about a large decline of bees worldwide which in turn could have significant effects on biodiversity and crop production (Kluser and Peduzzi 2007, Goulson *et al.* 2015, Tiardo *et al.* 2013, Novais *et al.* 2016). However, pollinator health is a highly complex topic that is not only driven by one but various factors which could act simultaneously (AFSSA 2009) and potentially synergize each other.

Especially insecticides have been occasionally accused to be a key driver in pollinator decline and compromised bee health (Goulson *et al.* 2015, Woodcock *et al.* 2017). Since insecticides are designed to target insects, it is not surprising that they could possibly have an impact on bee pollinators, too. However, the potential exposure of bee pollinators to insecticides does not necessarily pose a risk to them. The potential effects of crop protection products on bee pollinators are assessed by the manufacturers in a risk assessment following a hierarchical tiered testing approach which is submitted to regulatory authorities for evaluation. In the EU, all crop protection products introduced to the market are strictly regulated under the regulation EC 1107/2009 and possess no unacceptable side effects on the environment when they are used in accordance with the label instructions.

The bee safety of crop protection products is a key requirement in the development and (re)registration of products. The honey bee *Apis mellifera* is the predominately used bee species to assess the effects of crop protection products in the laboratory and under semi-field or field conditions. Although the acute intrinsic oral and contact toxicity for all commercial products to honey bees has been comprehensively assessed within the respective regulatory framework, less is known about the mechanisms in honey bees providing tolerance to crop protection products. Therefore, understanding the biochemical and molecular interactions of these products with honey bees and other bee pollinators is important to support the targeted design of insecticides with a low intrinsic toxicity to bees and thereby avoid non-target effects.

5.1 Cytochrome P450 monooxygenases of the honey bee and their role in insecticide metabolism

Cytochrome P450 monooxygenases (P450s) are hemoproteins and well-known for their role in the oxidative metabolism of insecticides in insects (Feyereisen 1999, Li *et al.* 2007). Especially P450s belonging to CYP clade 3 have been frequently identified as key determinants in insecticide resistance in pest species towards various chemical classes (Karunker *et al.* 2008, Bass *et al.* 2011, Zimmer *et al.* 2014, Pavlidi *et al.* 2018).

Compelling evidence that also honey bee P450s play a crucial role in the metabolism of insecticides was published by Iwasa and colleagues in 2004. They conducted synergist bioassays with known P450 inhibitors (Piperonyl butoxide (PBO) and certain fungicides) and demonstrated that these enzymes are crucial in the detoxification of *N*-cyanoamidine neonicotinoid insecticides as the acute contact toxicity of these compounds increased as a result of P450-inhibition (Iwasa *et al.* 2004). Further evidence that P450s are key drivers in the metabolism of neonicotinoid insecticides was obtained by analyzing the metabolite profile of [^{14}C]-labelled imidacloprid and acetamiprid *in vivo* after oral exposure in honey bees (Suchail *et al.* 2004b, Brunet *et al.* 2005) and the *in vivo* metabolism of imidacloprid (Suchail *et al.* 2004a). Additionally, the significant impact of honey bee P450s on the tolerance to pyrethroid insecticides has been illustrated by synergist bioassays, too (Johnson *et al.*2006).

Owing to the fact that genomic data was not available before 2006, it was not possible to study the function of individual honey bee P450s. In 2006, the honey bee genome was published and revealed the presence of 46 P450s clustering in four clades: 4 P450s were assigned to clade 4, 28 P450s to clade 3, 8 to clade 2 and 6 to the mitochondrial clade; respectively (Honey Bee Sequencing Consortium 2006; Claudianos *et al*. 2006).

At first glance, it was a surprising observation that honey bees own less detoxification enzymes compared to other insect species. In total, 10 glutathione S-transferases (GSTs), 24 carboxylesterases (CCEs) and 46 P450s were annotated in the honey bee genome (Honey Bee Sequencing Consortium 2006), a number that is significantly lower compared to other insect species such as *Drosophila melanogaster* (38 GSTs, 85 P450s and 35 CCEs) or *Anopheles gambiae* (31 GSTs, 106 P450s and 51 CCEs) (Claudianos *et al.* 2006). It was speculated that the obvious lack of detoxification enzymes in the honey bee could be reflected in an increased susceptibility towards insecticides (Claudianos *et al.* 2006). This assumption was confounded by a comparative analysis of the toxicity of 62 insecticides belonging to 6 chemical classes (carbamates, neonicotinoids, organochlorines, organophosphates, pyrethroids and miscellaneous) on honey bees, which demonstrated that honey bees are not more sensitive to insecticides than other insect species (Hardstone and Scott 2010).

However, today the knowledge on the interaction of insecticides with honey bees is mainly derived from *in vivo* bioassays, (Gong and Diao 2017). Biochemical and metabolism data is lacking in most cases but would help to better understand the detoxification capacity of honey bees towards crop protection products. Despite the availability of genome data and methods to functionally express P450s (Zimmer *et al.* 2014), the knowledge on the role of individual P450s involved in the oxidative metabolism of insecticides today derives from one study describing P450s belonging to the CYP9Q-subfamily in the oxidative metabolism of the in-hive used miticides coumaphos and *tau*-fluvalinate (Mao *et al.* 2011)

Consequently, further investigations are of great importance to address and close knowledge

gaps on the biochemical and molecular interactions of insecticides with honey bees and other bee pollinators.

5.2 Native microsomes as a tool to study P450-driven metabolism of insecticides and how to make them work

On the cellular level, P450s are located in the membranes of the smooth endoplasmic reticulum (ER) in eukaryotic cells (except mitochondrial P450s) and they can be easily isolated as native microsomes after mechanical destruction of the cells followed by several high speed-centrifugation steps (Claude 1969, Feyereisen *et al.* 1985). As the microsomal membranes contain numerous P450s acting in concert, they are a powerful *in vitro* tool to study the interactions of P450s with xenobiotics as well as to characterize the detoxification capacity of P450s with various substrates.

Having said this, the isolation of functional microsomal fractions from honey bees has been challenging in the past, whereas the preparation of these subcellular fractions is straightforward in other insect species (Feyereisen *et al.* 1985, Lee and Scott 1989, Scott 1996). A few publications have been dedicated to this topic and outlined the difficulties to obtain functional honey bee microsomes starting with Gilbert and Wilkinson in 1974. The researchers failed to isolate functional microsomes from various homogenized organs and tissues, but could measure a decent epoxidase, hydroxylase and O-demethylase activity in intact midguts of worker bees, drones and subcellular fractions of drone larvae (Gilbert and Wilkinson 1974). They assumed that a macromolecule located in the gut inhibited microsomal activity and published further experimental work indicating that the nucleic acid moiety of a macromolecule isolated from the soluble fraction of midgut preparations might be the factor leading to the inhibition (Gilbert and Wilkinson 1975). However, the inhibitor could not be further specified. A comparative study of the detoxification capacity of honey bee P450s, ESTs and GSTs was published in 1984 which confirmed the observations made by Gilbert and Wilkinson (Yu *et al.* 1984). Yu *et al.* (1984) failed to measure P450 activity in microsomes isolated from intact midgut preparations of worker bees and detected a large amount of P420, the intact form of P450s, in the respective microsomal fraction by determining the CO difference spectra. Therefore, all further experiments were conducted using intact midgut preparations rather than microsomes resulting in the measurement of P450 activity for the substrates that were analyzed. A direct comparison of honey bee P450, EST and GST activity levels to those measured in important pest species showed comparable catalytic rates for the analyzed substrates (in some cases higher, in some lower) (Yu *et al.* 1984). This observation further underlined the presence of effective enzymatic systems in honey bees to potentially degrade xenobiotics.

Since then, no firm work on the potential optimization of honey bee microsomes for P450-mediated detoxification explorations has been published, but further *in vitro* studies using either

intact honey bee midguts (Smirle and Winston 1987, Smirle 1993), microsomes prepared from dissected intact midguts, and/or fat body tissue from adults and larvae (Vidau *et al.* 2011, Fine and Mullin 2017) or floating abdomens (Alptekin *et al.* 2015, Todeschine *et al.* 2017).

Today, native microsomes are again gaining greater interest as a tool to study the oxidative detoxification capacity of honey bees *in vitro*. In this thesis, the problems associated with the isolation of functional microsomes from honey bees were approached (Zaworra and Nauen 2019, chapter 2). As the dissection of worker bee midguts is quite time-consuming, the objective was to develop a method to prepare microsomal fractions based on whole abdomens. The first attempts to isolate functional microsomes from worker bee abdomens failed and no P450-activity could be measured with a range of coumarin-based fluorescence model substrates (Zaworra and Nauen 2019, chapter 2), thus confirming earlier results (Gilbert and Wilkinson, 1974 and 1975, Yu *et al.* 1984). Female honey bees own a sting attached to a venom sac for defensive purposes (Nouvian *et al.* 2016), so it is not unlikely that the observed inhibition of microsomal activity in abdominal preparations might be due to the presence of venom sac toxins. The honey bee venom is a complex composition of various molecules, especially peptides and proteins (Banks and Shipoloni 1986, Dotimas and Hilder 1987, Peiren *et al.* 2005) which could possibly interfere with the activity of P450s. Ultimately, functional microsomal fractions from whole worker honey bee abdomens were obtained by simply removing the venom gland sting complex using forceps prior to tissue homogenization (Zaworra and Nauen 2019, chapter 2). The enzyme activity was confirmed with a subset of 13 model substrates and revealed that microsomal P450s show a strong preference for coumarin-based fluorogenic model substrates over resorufins, with the highest turnover of 7-benzyloxy-4-trifluromethyl coumarin (BFC). Besides the enzymatic confirmation, the respective CO difference spectra of microsomal preparations indicated the presence of a high amount of functional P450s in fractions prepared with prior removal of the venom gland sting complex (Zaworra and Nauen 2019, chapter 2).

Additionally, the honey bee venom was further characterized providing compelling evidence that phospholipase A_2 (PLA_2) is the factor responsible for the inactivation of microsomal P450 activity (Zaworra and Nauen 2019, chapter 2), most-likely by PLA_2 hydrolyzing the 2-acyl bonds of glycerophospholipids of the microsomal membranes (Habermann 1972, Dennis 1991) which have to be undamaged for the proper function of these membrane-bound hemoproteins.

In the study outlined in chapter 2 (Zaworra and Nauen 2019), native microsomes were prepared from worker bees that were taken from the hive and anaesthetized with CO_2. During the removal of the venom gland sting complex, the secretion of venom out of the sting was often observed although the bees were anesthetized. Since Gilbert and Wilkinson (1974) and Yu *et al.* (1984) were not able to isolate functional microsomes from worker midgut preparations, it is rather likely that their preparations were contaminated with PLA_2 leading to the disintegration

of microsomal membranes. One indicator supporting this hypothesis is the measurement of a high amount of P420 and almost no P450 in CO-difference spectra as reported by Yu *et al.* (1984). However, no detailed information about the preparation procedure is given by the authors, but it is highly likely that the digestive system was removed by pulling it out with forceps from the tip of the abdomen and thus contaminations could occur rapidly and unnoticed. The present study outlines a method for the successful isolation of native microsomes from honey bee abdomens, but also delivers a proof of concept by demonstrating the ability of microsomal P450s to degrade certain neonicotinoid insecticides by hydroxylation for the first time (Zaworra and Nauen 2019, chapter 2). The hydroxylation of the *N*-cyanoamidine thiacloprid by microsomal P450s *in vitro* was significantly higher compared to the *N*-nitroguanidin imidacloprid, thus providing evidence for a different detoxification capacity towards neonicotinoid insecticides.

Although the removal of the venom gland sting complex before tissue homogenization is still laborious, it is less time consuming compared to the preparation of intact midguts and allows to conduct *in vitro* studies with honey bee microsomes with flexibility and off season as the enzyme activity has been proven to remain stable in frozen microsomal fractions for about six months (Zaworra and Nauen, chapter 2).

5.3 P450s as key determinants of bee-sensitivity to neonicotinoid insecticides

Neonicotinoids are systemic insecticides which are widely used for the control of sucking and certain chewing pest species in various crops (Jeschke and Nauen 2008). As described in the introductory chapter of this thesis, the acute intrinsic toxicity of neonicotinoids to honey bees differs significantly, i.e. the *N*-cyanoamidines thiacloprid and acetamiprid are over 2-3 orders of magnitude less toxic compared to the *N*-nitroguanidine representatives such as imidacloprid (Iwasa *et al.* 2004).

One aim of this thesis was to provide evidence for the presence of biochemical and molecular defense mechanisms explaining the lower acute toxicity of *N*-cyano-substituted neonicotinoids compared to *N*-nitro-substituted compounds in the honey bee (Manjon *et al.* 2018, chapter 3). Additionally, it was of interest to answer the same question for the bumblebee *B. terrestris* as another project part of the "Bee Toxicogenomics Project", since a similar toxicity range was determined (Manjon *et al.* 2018, chapter 3).

Radioligand binding studies using head membrane preparations revealed that the difference in toxicity does not reside at the molecular target as both compounds, thiacloprid and imidacloprid, showed a similar nanomolar binding affinity to postsynaptic nicotinic acetylcholine receptors (nAChRs) of the honey bee and bumblebee (Manjon *et al.* 2018, chapter 3). This observation, together with previously published synergist studies (Iwasa *et al*. 2004), as well as further ones conducted for thiacloprid and imidacloprid on both bee species strongly suggested a metabolic

origin that is underlying the bee-sensitivity of neonicotinoid insecticides (Manjon *et al.* 2018, chapter 3). Great efforts were made in order to functionally express all honey bee P450s belonging to the monophyletic clade 3. The individual incubation of P450s with thiacloprid and imidacloprid in presence of $NADPH^+$ coupled to UPLC-MS/MS analysis of the respective 5`-hydroxy-metabolite identified P450s belonging to CYP9Q-subfamily as the key determinants mediating bee-sensitivity to neonicotinoid insecticides. CYP9Q3 was identified as the particular key enzyme involved in the rapid turnover of thiacloprid in the honey bee, but with significantly lower activity against imidacloprid. Additionally, the respective orthologue P450s of the bumblebee, CYP9Q4 and CYP9Q5, were identified in the metabolism of thiacloprid. CYP9Q4, showed a significant higher turnover of thiacloprid compared to imidacloprid (Manjon *et al.* 2018, chapter 3). Ultimately, this finding was confirmed by transgenic *D. melanogaster* lines ectopically expressing the individual honey bee and bumblebee P450s resulting in resistance to thiacloprid when compared to flies with the same genetic background not expressing the bee P450. Both, honey bee and bumblebee P450s belonging to the CYP9Q-family were also identified to be involved in the metabolism of acetamiprid, but with an overall lower turnover compared to thiacloprid (Manjon *et al.* 2018, chapter 3).

This study illustrates that low acute toxicity of *N*-cyanoamidine neonicotinoid insecticides to honey bees and bumblebees is strongly driven by individual P450s which were found to be highly expressed in the brain, Malpighian tubules and midgut of both bee species (Manjon *et al.* 2018, chapter 3). This finding is remarkable and does not only provide further knowledge on the detoxification capacity of bee P450s, but also emphasizes the importance of P450-mediated detoxification as a biochemical defense mechanism in encountering certain neonicotinoid insecticides and thereby providing tolerance to honey bees and bumblebees when foraging on treated crops.

5.4 The contribution of pharmacokinetic parameters to the acute intrinsic toxicity of neonicotinoid insecticides in honey bees after contact exposure

The fate of a molecule within an organism is not only described by the metabolization capacity alone, but by the different pharmacokinetic parameters also summarized as ADME (absorption, distribution, metabolism and excretion). So far, three studies have been published analyzing the fate of the neonicotinoid insecticides imidacloprid and acetamiprid after oral exposure and revealed a rapid metabolism after ingestion of the respective compound (Suchail *et al.* 2004a, Suchail *et al.* 2004b; Brunet *et al.* 2005). However, knowledge on the fate of these neonicotinoids upon contact exposure is lacking and in case of thiacloprid, both routes of exposure have not been studied at all.

The consideration of the contact route of exposure is important as honey bees or other bee pollinators may get in contact with residues of crop protection products by direct overspray or

while collecting pollen from treated plants. The insect's cuticle is a complex extracellular layer forming the exoskeleton and serves as a barrier between the insect and the environment. Upon contact, a molecule has to pass a lipophilic-hydrophilic system, consisting of the outer waxy lipophilic epicuticle followed by the hydrophilic inner procuticle. Thus, the penetration of a molecule through this protective layer is influenced by the physiochemical properties of the molecule itself and could be further enhanced by solvents used for application (Yu 2008).

In order to close the knowledge gap on the mode of entry, a method to track [^{14}C]-labelled neonicotinoid insecticides upon contact exposure was established (Zaworra *et al.* 2019, chapter 4). Pharmacokinetic parameters were assessed at different elapsed time intervals over 24 h after contact application of [^{14}C]-thiacloprid (TCP), [^{14}C]-acetamiprid (ACT) and [^{14}C]-imidacloprid (IMD) onto the dorsal thorax of honey bees.

TCP showed the lowest penetration rate in line with highest metabolization and elimination rate of the three analyzed neonicotinoid insecticides. For the first time, the *in vivo* metabolism of TCP was elucidated by the detection of hydroxy-TCP, its corresponding amide, an unknown metabolite and traces of the TCP-amide metabolite in honey bee extracts. Moreover, 6-chloronicotinic acid (6-CNA) was described as a common metabolite for all analyzed neonicotinoid insecticides. ACT showed a similar penetration pattern, but a lower turnover rate when compared to TCP (Zaworra et al. 2019, chapter 4). The elucidated metabolite profile of ACT was congruent with a previously published study examining the metabolic fate after oral exposure to [^{14}C]-ACT (Brunet *et al.* 2005). With regard to the contribution of individual P450s in the detoxification of both compounds, the higher turnover of TCP *in vivo* is indicated to be mainly driven by CYP9Q3 resulting in a lower intrinsic toxicity to honey bees upon contact exposure (LD_{50}: 14.6 µg a.i./bee) compared to ACT (LD_{50}: 7.07 µg a.i./bee) (Iwasa *et al.* 2004) which is mainly metabolized by CYP9Q2 but with an overall lower turnover compared to TCP (Manjon *et al.* 2018, chapter 3).

Due to the high intrinsic toxicity of IMD, honey bees were exposed to 100x lower doses compared to *N*-cyano-substituted neonicotinoid insecticides. Since the applied IMD dose was slightly below the LD_{50}, the bees showed strong symptoms of poisoning. This dose was necessary to meet the threshold for detecting the [^{14}C]-radiolabel. IMD showed the fastest penetration rate with half of the applied dose penetrated through the cuticle within 24 h resulting in high internal body concentrations when compared to TCP and ACT. Again, the metabolic fate of IMD was in line with a previously published study examining the fate of [^{14}C]-IMD after oral exposure (Suchail *et al.* 2004b). Hydroxy-IMD, 4,5-dihydroxy-IMD and IMD-olefine were the main metabolites identified in honey bee homogenates 24 h after contact exposure. Both metabolites still carry the nitro-pharmacophore and such metabolites have been demonstrated to own a similar intrinsic toxicity as the parent compound itself (Nauen *et al*. 2001), thus leading to further intoxication. Additionally, the results suggest that also the significantly lower

CYP9Q-driven detoxification of IMD is contributing to the higher intrinsic activity of this compound to honey bees.

This study provided novel insights in the pharmacokinetics as a component contributing to the lower intrinsic toxicity of *N*-cyanoamidine neonicotinoids after contact exposure in the honey bee and complements the investigations on the importance of P450-driven detoxification of neonicotinoid insecticides (Zaworra and Nauen 2019, Manjon *et al.* 2018, chapter 3).

5.5 Practical relevance of the reported biochemical and molecular findings

The potential adverse effects of crop protection products, especially neonicotinoid insecticides, on bee pollinators have been addressed in publications on an almost weekly basis (Sgolastra *et al.* 2018, Otesbelgue *et al.* 2018, Crall *et al.* 2018). The experimental work underlying these studies were mostly generated in the laboratory aiming to mimic "field-realistic" conditions and often the adverse effects of crop protection products observed in these studies do not occur in higher tier and field studies. *N*-nitroguanidine neonicotinoid insecticides for instance display a high acute toxicity on honey bees, which can be easily shown in acute laboratory studies whereas no adverse effects were found on the colony level in various conducted field studies under applied conditions (Cutler and Scott-Dupree 2007, Pohorecka *et al.* 2012, Pilling *et al.* 2013, Cutler *et al*. 2014, Rolke *et al.* 2016).

To quote Paracelsus "*sola dosis facit venenum*" - when it comes to the toxicity of any existing molecule, the dose makes the poison. The biochemical and molecular mechanisms resulting in the tolerance of insecticides to bee pollinators has not been closely investigated for a number of chemical classes, yet. The toxicogenomic studies outlined in this thesis address this knowledge gap and shed light on the crucial role of bee P450s as key mediators of bee-sensitivity to neonicotinoid insecticides (Manjon *et al.* 2018, chapter 3). Moreover, pharmacokinetic parameters were described as important factors contributing to the intrinsic toxicity of a compound upon contact exposure in honey bees (Zaworra *et al.* 2019, chapter 4).

But what is the impact from an applied perspective?

If honey bees are topically or orally exposed to environmentally relevant doses of *N*-cyanoamidine neonicotinoid insecticides they are likely to encounter in the field, honey bees are able to rapidly break down these compounds by P450-driven oxidative metabolism. This explains the bee-safe field use of the respective products when they are applied in accordance with the label instruction.

In this thesis imidacloprid was studied as a representative for *N*-nitroguanidine neonicotinoid insecticides. It was demonstrated that CYP9Q-enzymes also detoxify IMD, although the turnover was significantly lower compared to TCP. The fact that no adverse effects of *N*-nitroguanidine neonicotinoid insecticides on the colony level were observed in most field studies (Cutler and Scott-Dupree 2007, Pohorecka *et al.* 2012, Pilling *et al.* 2013, Cutler *et al*.

2014, Rolke *et al.* 2016) could be explained by the detoxification capacity of these P450s towards field-realistic residues enabling the honey bees to overcome the toxicity of the compounds.

Neither today nor in the near future biochemical and molecular data will likely be part of the regulatory data package for product registration since the knowledge insufficient. Nevertheless, the methods established in this thesis could be supportive to gain a mechanistic understanding of the interaction of crop protection products with bee pollinators. To give one applied example, biochemical investigations on synergistic effects between different compounds could be of great interest in the context of product development and safety assessment. For instance, certain fungicides inhibiting ergosterol biosynthesis have been described as synergists when applied in combination with certain insecticides resulting in a higher intrinsic toxicity to bees (Pilling and Jepson 1993, Iwasa *et al.* 2004, Raimets *et al.* 2017). Manufacturers of crop protection products consider potential synergism of co-applied products in the risk assessment during the registration process of their products. The analysis of these interactions is of high importance as farmers often treat their crops with a mixture of different compounds simultaneously. Substrate depletion assays could offer an applied solution to analyze synergistic interactions beyond *in vivo* ecotoxicological studies, if the key enzymes involved in the detoxification of an insecticide of interest are known. In principle, the enzyme involved in the oxidative metabolism of the compound of interest is functionally expressed and afterwards incubated with a fluorescence substrate in presence and absence of the potential synergist. The decrease of the fluorescence readout in comparison to a reaction catalyzed in the absence of the potential synergist would indicate the synergistic potential and inhibitory strength of a molecule which is then proportional to the magnitude of florescence depletion. If for instance the P450-activity would be strongly inhibited by an azole fungicide, the insecticide cannot be sufficiently metabolized anymore resulting in increased toxicity levels. Thus, the observed synergism of the azole fungicides triflumizole and propiconazole when co-applied with thiacloprid (Iwasa *et al.* 2004) is a result of the inhibition of CYP9Q-enzymes resulting in significantly elevated toxicity levels. In this regard, biochemical data could be useful for screening purposes or to support the interpretation of results from laboratory and field studies.

In addition, the transgenic *Drosophila melanogaster* lines ectopically expressing the respective key honey bee and bumblebee P450s involved in the rapid detoxification of *N*-cyanoamidine neonicotinoid insecticides (Manjon *et al.* 2018, chapter 3) offer an interesting *in vivo* tool to answer questions on the detoxification capacity of these key enzymes towards other chemical classes. Since the work on honey bees is seasonally restricted, this system offers the benefit to rear the transgenic flies for testing purposes all year round.

In the past, it was speculated that the lower number of detoxification enzymes in the honey bee could be reflected by an increased susceptibility towards insecticides (Claudianos *et al.* 2006). The outlined work highlights the important role of individual honey bee P450s as mediators of differential toxicity to neonicotinoid insecticides and thus underlines the importance of this enzyme family in encountering xenobiotics - regardless of their total number.

5.6 Future work perspectives

Unravelling the role of honey bee P450s as key determinants mediating bee-sensitivity to neonicotinoid insecticides was part of a holistic approach to understand insecticide bee pollinator interactions at the molecular and biochemical level. The established biochemical and molecular methods are ready to be use to address fundamental research questions as well as applied questions in the bee safety evaluation of crop protection products.

Future work on the metabolism of a broad spectrum of chemical classes from synthetic and natural origin would be of interest to obtain a clearer picture on the detoxification capacity of individual honey bee P450s and their preference to catalyze certain chemical structures. This knowledge is of high value for the targeted design of insecticides with a low intrinsic toxicity to bees. Furthermore, the knowledge could be implemented in *in silico* screening tools to predict insecticide metabolism.

Moreover, the methods to study P450-driven metabolism *in vitro* could be implemented as medium to high throughput assays, e.g. to support the early chemistry screening cascade with regard to bee-selectivity of new molecules. Native microsomes, i.e. liver microsomes or individual P450s (Jia and Liu 2007, Harper and Brassil 2008) were established as a standard screening assay in the drug screening cascade in the pharmaceutical industry.
In terms of the methodology, the incubation of individual or microsomal honey bee P450s with a compound of interest coupled to analytical verification would be straightforward, but for large scale screening still laborious and time-consuming. The *in vitro* analysis of the depletion of model substrate fluorescence by individual or microsomal P450s in the presence of crop protection products would offer a rapid solution. Microsomal as well as certain individual P450s showed a strong preference for coumarin-based artificial model substrates over resorufins (Manjon *et al.* 2018, chapter 3, Zaworra and Nauen 2019, chapter 4). BFC has been identified as an appropriate substrate metabolized by microsomal and individual P450s (Manjon *et al.* 2018, chapter 3, Zaworra and Nauen 2019, chapter 4) and could serve as a starting point for the development of a high throughput screening method based on fluorescence depletion. Additionally, the optimization of the preparation procedure of native honey bee microsomes for high throughput purposes would be required taking into consideration the possible destructive

effects of PLA_2.
In general, thresholds that allow the extrapolation of these *in vitro* results to the intrinsic *in vivo* toxicity of a compound to bees would need to be established.

The outlined findings, taken together with previously published data, strongly indicate wider enzymatic properties of the CYP9Q-family towards the metabolism of insecticides. In this regard, it is tempting to speculate that certain honey bee P450s, such as the CYP9Q-subfamily, might have a stronger contribution to detoxification processes than other P450s. In humans for instance, CYP3A4 expressed in the liver tissue is the major P450 responsible for the turnover of most drugs (Wilkinson 2005). The honey bee CYP9Q-enzymes have now been described in the metabolism of three chemical classes, organophosphates and pyrethroids (Mao *et al.* 2011) and neonicotinoids (Manjon *et al.* 2018, chapter 3), as well as the phytochemical quercetin (Mao *et al.* 2011), with an outstanding detoxification capacity observed for honey bee CYP9Q3. Interestingly, gene expression of CYP9Q3 was reported to be induced after contact with p-coumaric acid, a phytochemical commonly found in bee-relevant matrices such as nectar and pollen (Mao *et al.* 2013). Moreover, the high expression of CYP9Q3 in worker bee hind legs, the place where pollen is collected and transported in so called corbiculae, have been reported (Mao *et al.* 2015) and indicate a ubiquitous importance of this particular P450 in encountering exposure to various xenobiotics. As forager bees are usually the first individuals possibly exposed to xenobiotics while foraging, this hypothesis is also supported by the observation that the expression of CYP9Q3 in the mandibular glands of forager bees is higher compared to nurse bees (Vannette *et al.* 2015). Moreover, CYP9Q3 was found to be highly expressed in the Malpighian tubules, midgut and brain of worker bees (Manjon *et al.* 2018, chapter 3). To obtain a deeper knowledge on the role of P450s belonging to the CYP9Q-subfamily, the broader screening of various molecules from synthetic and natural origin would be the logical next step.

In this regard, the outlined toxicogenomic tools could be applied to study the interaction of P450s and plant-derived allelochemicals to address questions in fundamental research. Today, the highly-complex interaction of honey bees with allelochemicals is not well understood in both, ecological function and the mechanisms providing tolerance to certain compounds (Detzel and Wink 1993, Stevenson *et al.* 2017). On the molecular basis, certain clade 3 P450s have been described in the metabolism of the flavonoid quercetin so far (Mao *et al.* 2009, Mao *et al.* 2011) and there is experimental evidence that P450s play a key role in the metabolism of nicotine, too (Du Rand *et al.* 2015, Du Rand *et al.* 2016). However, the respective key enzyme(s) facilitating oxidative metabolism of nicotine remain unknown so far. This raises the question to what extent CYP clade 3 enzymes might be also involved in the broader detoxification of allelochemicals.

Future investigations applying molecular and biochemical tools would thus be of interest to gain insights on the interaction of bee pollinators with flowering plants.

The presented toxicogenomic investigations were conducted on managed bee species with different degrees of sociality, *A. mellifera* and *B. terrestris*, respectively. To be able to extrapolate the finding to other bee species, i.e. solitary bees or to other social bee groups like stingless bees, it would be of great importance to have annotated genome data in a good quality available.

Another major achievement of the "Bee Toxicogenomics Project" was the characterization of the molecular determinants of neonicotinoid sensitivity to the red mason bee *Osmia bicornis*. Similar to honey bees and bumblebees, the *N*-cyano-substituted compound thiacloprid display a significantly lower acute intrinsic toxicity to the red mason bee compared to the *N*-nitro-substituted compound imidacloprid and also in this bee species, the differential toxicity was described not to derive from differences in the binding affinity to nAChRs (Beadle *et al.*, 2019). Genome sequencing and annotation revealed that the *O. bicornis* CYPome lacks the CYP9Q-family, but owns orthologous P450s belonging to the CYP9BU-family which were elucidated as the key enzymes conferring tolerance to thiacloprid (Beadle *et al.*, 2019).

Overall, the acute intrinsic toxicity of certain neonicotinoid insecticides to this solitary bee species is comparable to the social species *A. mellifera* and *B. terrestris* and thus providing evidence for a conserved biochemical mechanism underlying the differential toxicity of neonicotinoid insecticides.

This observation raises the question to what extent the presence or absence of CYP9Q-orthologus in other bee species could serve as a predictive tool on species sensitivity towards certain insecticides. A recently published study compared the CYPomes of different bee species with regard to footprints of eusociality in phytochemical detoxification pathways (Johnson e*t al*. 2018). All examined species encoded a certain number CYP6AS-enzymes in their genomes. Moreover, one to four CYP9Q-genes were annotated in the respective genomes, except the complete lack of this subfamily in the alfalfa leafcutter bee *Megachile rotundata* (Johnson e*t al*. 2018). Studies on the particular P450s of these bee species would be of high interest to investigate to what extent the detoxification mechanisms are conserved among different species. Deeper knowledge on the intrinsic acute toxicity would complement these biochemical studies. However, the methods to assess the acute intrinsic toxicity of crop protection products on bee species other than the honey bee and bumblebee are still under development by ring testing groups so that a comparative *in vivo* analysis of the species sensitivity is not yet possible at the moment.

The knowledge on species sensitivity would also be of a high value to address questions in a changing regulatory environment for crop protection products. In the EU a novel risk assessment scheme has been proposed by the European Food Safety Authority (EFSA) outlining the evaluation of the effects of crop protection products on *Bombus* spp. and solitary bees as well as honey bees (EFSA 2013). In the EFSA bee Guidance Document *O. bicornis* and *O. cornuta* are proposed as representative species to assess the effects of crop protection products on solitary bees. However, the methods to assess the acute toxicity of compounds to *Osmia* spp. are currently still under development. Besides the lack of many requested testing methods, the EFSA Bee Guidance Document is based on overly conservative assumptions and demands higher tier testing methods that are not available (ECPA 2017). Thus deeper knowledge on species sensitivity could be of great contribution in the science-based discussion on the risk assessment paradigm for non-*Apis* species (Boyle *et. al*., Gradish *et. al*, 2019, Cham *et al.*, 2019).

Finally, growing knowledge on the detoxification capacity of honey bee P450s could be implemented in modelling approaches. Modelling programs such as BEEHAVE are developed for the *in silico* assessment of multiple stressors to a honey bee colony considering factors such as *Varroa* mite infestation, transmission of viruses, nutrition, landscape and pesticide-induced losses of honey bee castes either independently or in context of the landscape (Becher *et al.* 2014). The consideration of honey bee detoxification mechanisms would offer a valuable refinement option of the *in silico* model and thereby providing a higher level of certainty in modelling approaches.

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