Pesticide Biochemistry and Physiology 161 (2019) 68–76

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

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

ARTICLE INFO

Keywords:
Cytochrome P450
Detoxification
Honey bee
Neonicotinoid
Thiacloprid

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).

https://doi.org/10.1016/j.pestbp.2019.04.014
Received 26 March 2019; Received in revised form 8 April 2019; Accepted 29 April 2019
Available online 30 April 2019
0048-3575/

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

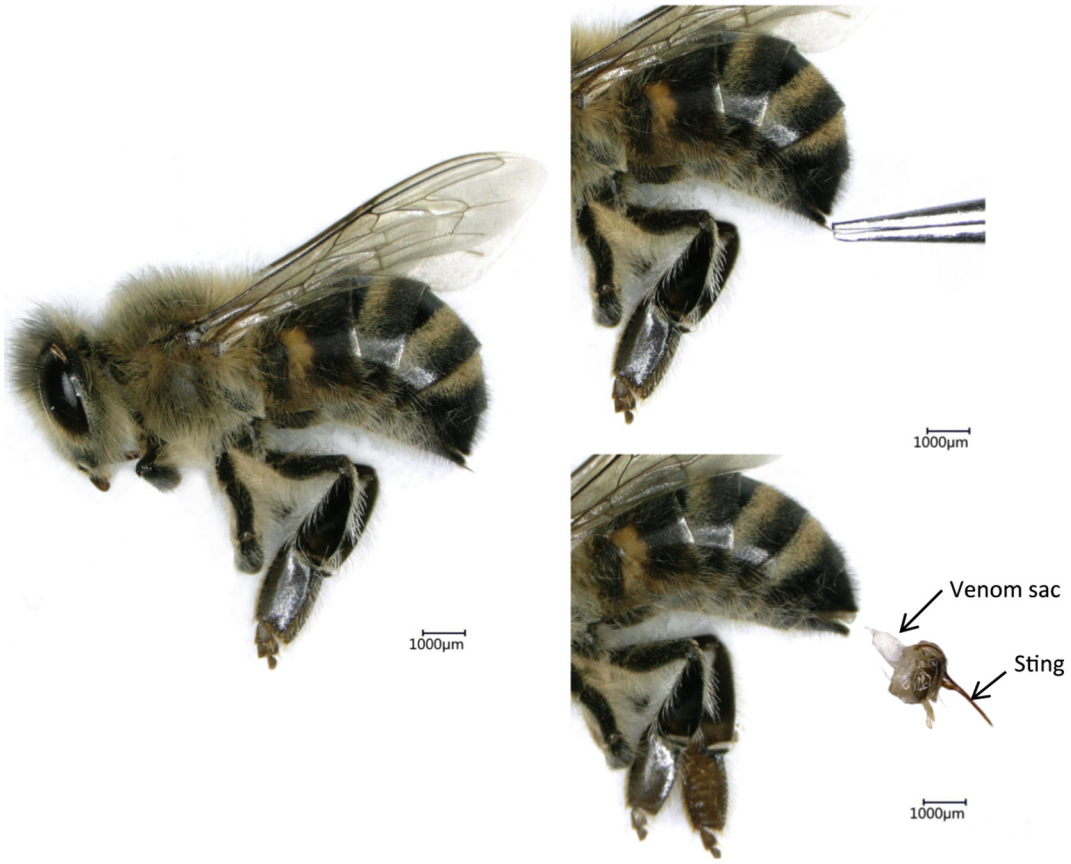

Fig. 1. Removal of the sting venom gland complex including the venom sac of a CO_2 anaesthetized honey bee using a pair of tweezers.

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.

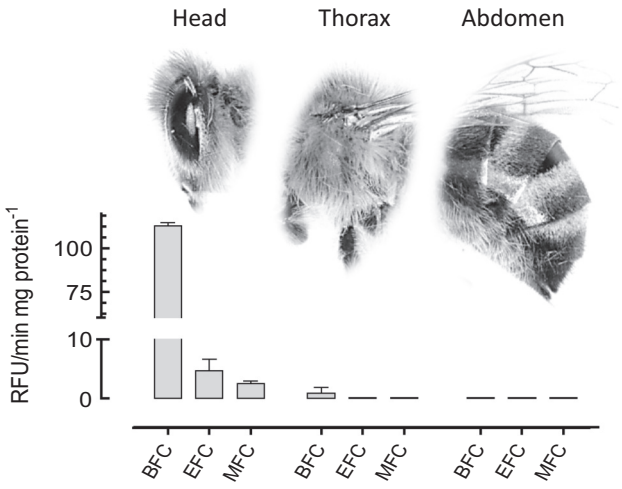

Fig. 2. Cytochrome P450 activity in microsomal preparations of different body parts of *Apis mellifera*. Enzyme activity is given in relative fluorescence units (RFU) based on incubations with different model substrates: 7-benzyloxy-4-trifluoromethyl coumarin (BFC), 7-ethoxy-4-trifluoromethyl coumarin (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.

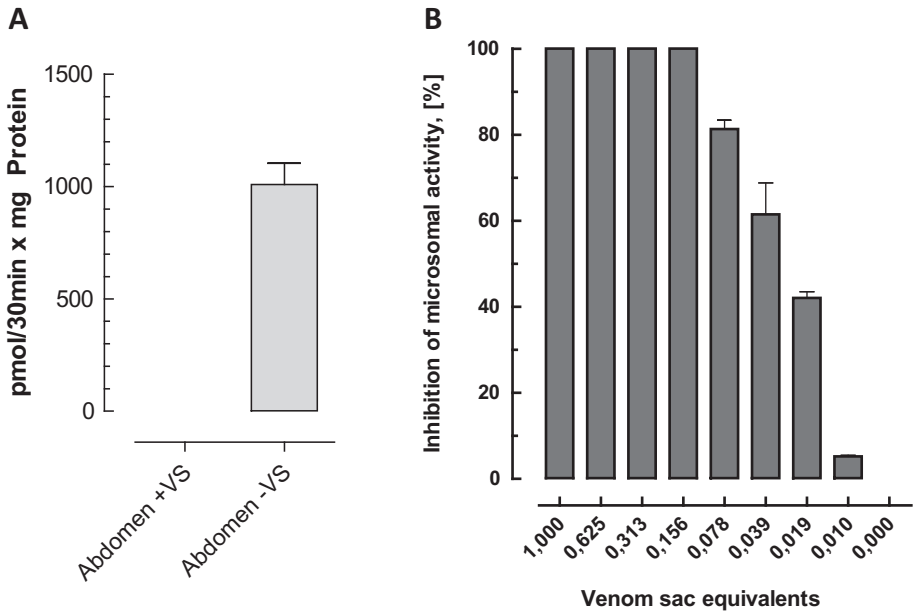

Fig. 3. (A) Cytochrome P450 activity using benzoxyfluorocoumarin as a substrate of honey bee micrsomes prepared from abdomens with (+*VS*) and without (-*VS*) venom sac. (B) Microsomal inhibition of 7-ethoxycoumarin O-deethylase activity in rat liver microsomes by serial dilutions of honey bee venom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

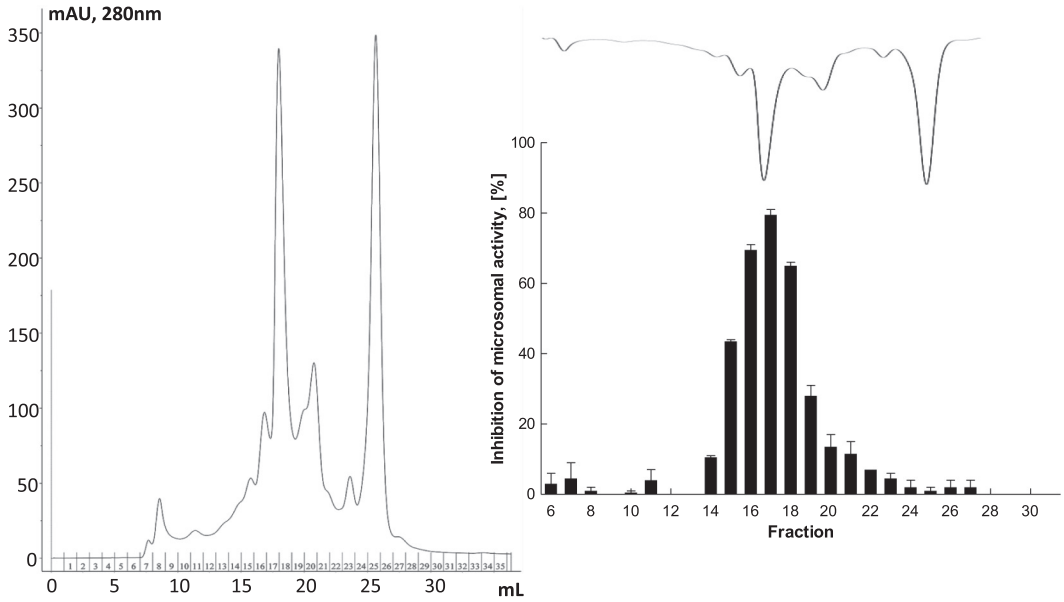

Fig. 4. Elution profile of honey bee venom sac proteins (1 mg) after size exclusion chromatography on Superdex S200 10/300 GL. The absorbance (mAU) was measured at 280 nm and 1 mL fractions were collected. Aliquots were subjected to a rat microsomal inhibition assay using 7-ethoxycoumarin.

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.

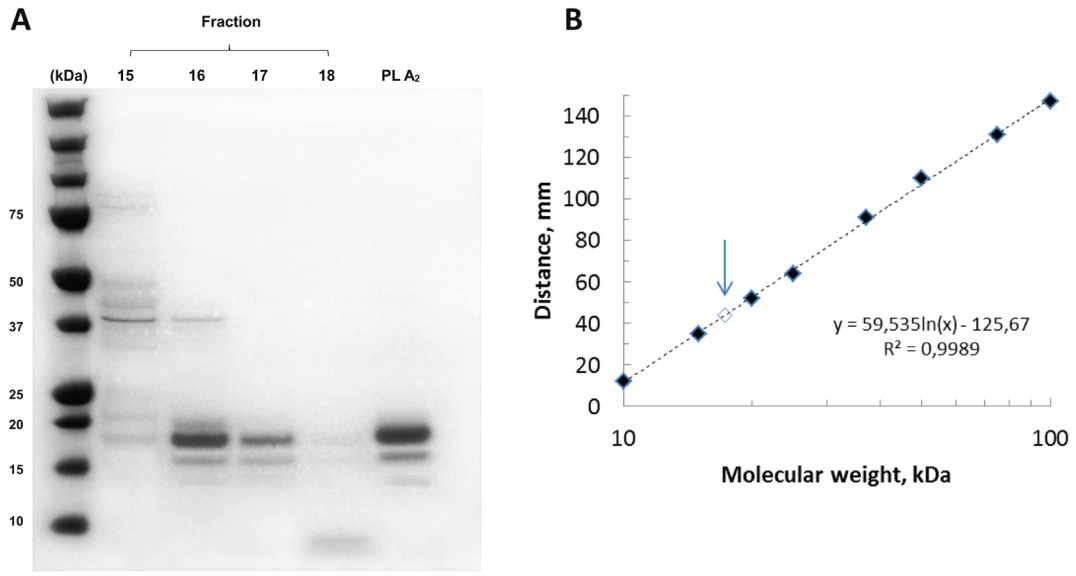

Fig. 5. (A) SDS PAGE of selected FPLC fractions of eluted honey bee venom sac proteins exhibiting the highest inhibitory activity on rat microsomal O-deethylation activity (EC). Molecular weight marker proteins are shown on the left, whereas a purified commercial sample of bee venom phospholipase A_2 (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.

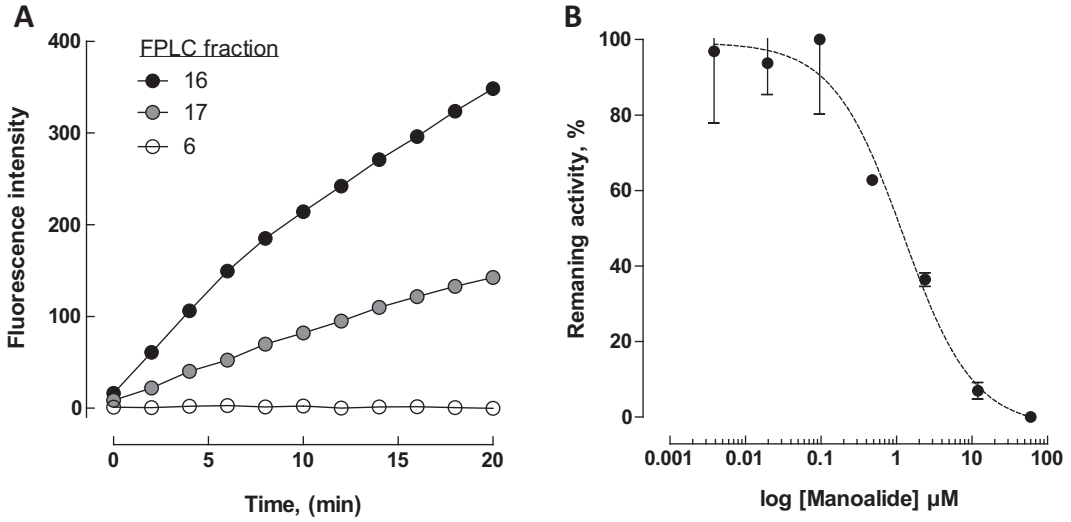

Fig. 6. (A) Measurement of phospholipae A_2 activity in FPLC fractions of honey bee venom collected after size exclusion chromatography on Superdex S200. (B) Inhibition by manoalide of honey bee phospholipase A_2 activity present in FPLC fraction #16.

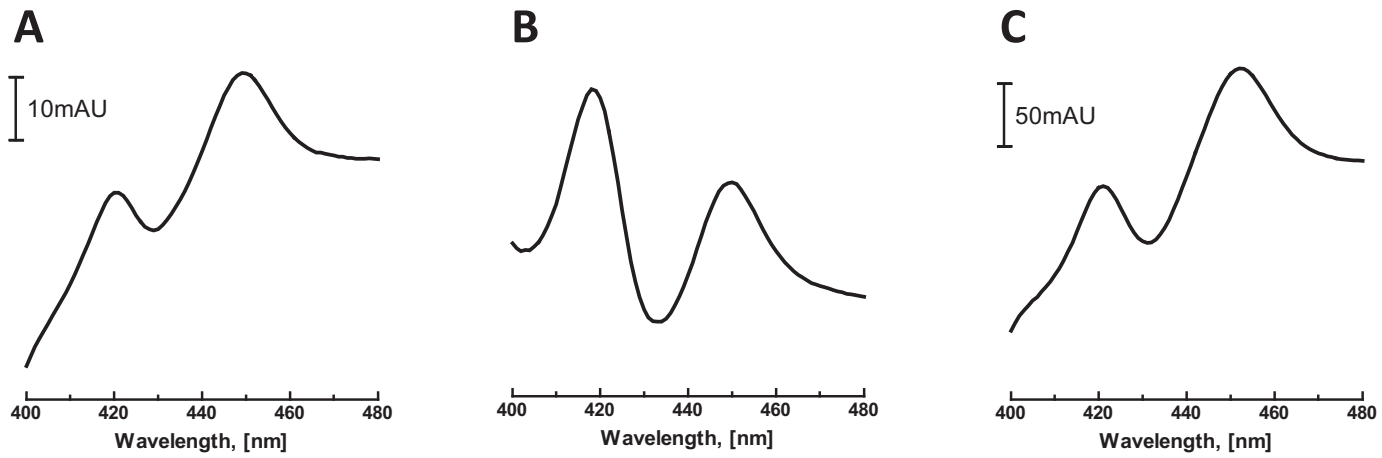

Fig. 7. CO-binding difference spectra recorded from microsomal preparations. (A) honey bee abdomens, venom sac removed; (B) honey bee abdomens, venom sac included; (C) rat liver.

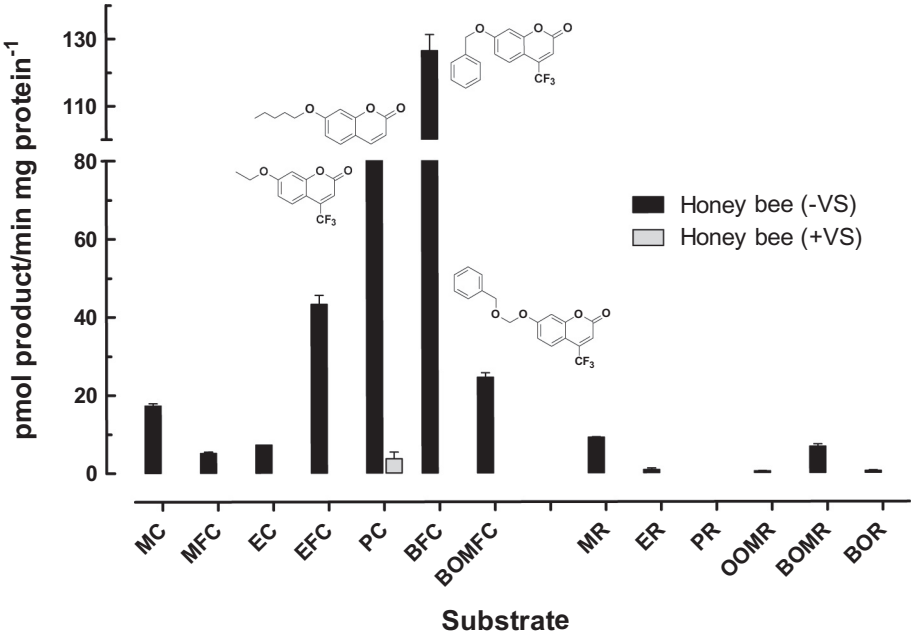

Fig. 8. Metabolism (O-dealkylation/−dearylation) of different fluorogenic coumarin and resorufin substrates by microsomal preparations obtained from honey bee (± *VS*, venom sac). Data are mean values ± SD (n = 3).
Abbreviations: BFC, 7-benzyloxy-4-trifluoromethyl coumarin; MFC, 7-methoxy-4-trifluoromethyl 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.

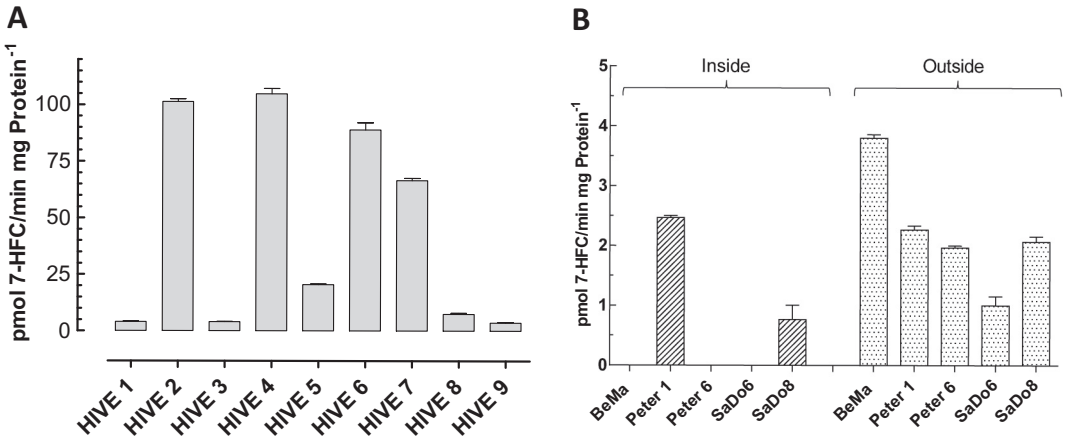

Fig. 9. (A) Variation in cytochrome P450 activity of microsomal preparations of mass homogenates of honey bees (n = 15) collected from combs of different hives (same day) using 7-benzyloxy-4-trifluoromethyl coumarin (BFC) as a substrate. (B) Variation of microsomal BFC O-dearylation activity of honey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

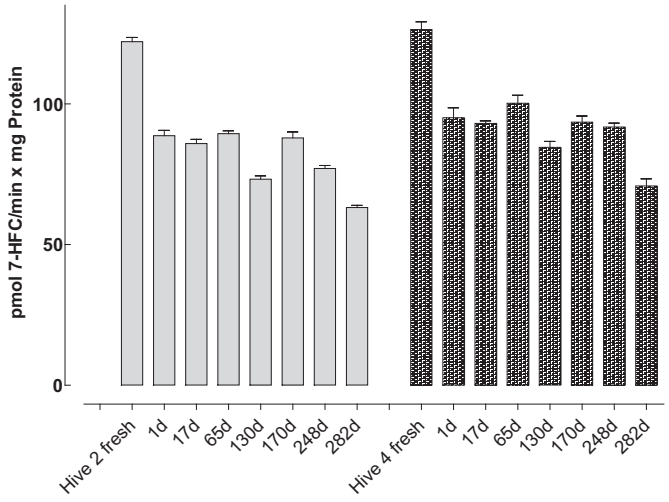

Fig. 10. Stability at −80 °C of microsomal preparations of honey bees collected from two different hives. The microsomal activity was measured after thawing at different elapsed time intervals using 7-benzyloxy-4-trifluoromethyl coumarin (BFC) as a substrate. Data are mean values ± SD (n = 3).

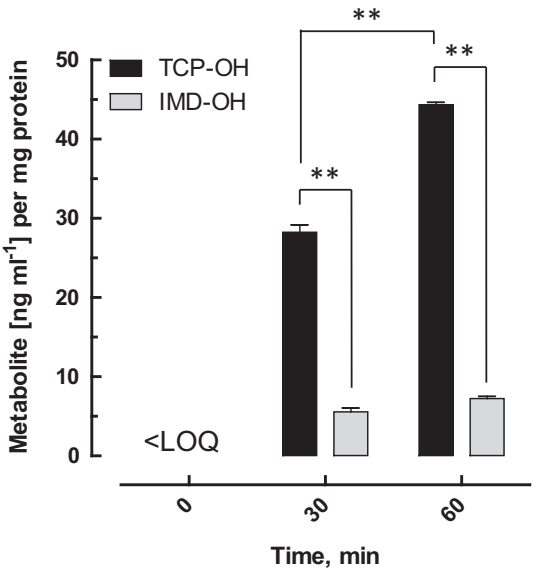

Fig. 11. Hydroxylation of thiacloprid (TCP) and imidacloprid (IMD) at different elapsed time intervals by microsomes prepared from honey bee abdomens after removal of the sting venom sac complex. Microsomes incubated without NADPH were inactive (data not shown). Data are mean values ± SD (n = 3); **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

Acknowledgements

We are grateful to Isabelle Marsch and Bettina Lueke for experimental support. Furthermore, we would like to acknowledge the analytical support provided by Martin Kaussmann and his team.

References

Agosin, M., Michaeli, R., Miskus, R., Nagasawa, S., Hoskins, W.M., 1961. A new DDT-metabolizing enzyme in the German cockroach. J. Econ. Entomol. 54, 340–342.
Alptekin, S., Philippou, D., Field, L., Wegorek, P., Zamojska, J., Moores, G., 2015. Insecticide synergists: good or bad for honey bees? Outlooks Pest Manag. 26, 75–77.
Altmann, F., Kubelka, V., Staudacher, E., Uhl, K., Maerz, L., 1991. Characterization of the isoforms of phospholipase A_2 from honey bee venom. Insect Biochem. 21, 467–472.
Arias, R.O., Terriere, L.C., 1962. The hydroxylation of napththalene-1-C^{14} by house fly microsomes. J. Econ. Entomol. 55, 925–929.
Balsinde, J., Balboa, M.A., Insel, P., Dennis, E.A., 1999. Regulation and inhibition of phospholipase A_2. Annu. Rev. Pharmacol. Toxicol. 39, 175–189.
Banks, B.E.C., Shipoloni, R.A., 1986. Chemistry and pharmacology of honey-bee venom. In: Piek, T. (Ed.), Venoms of the Hymenoptera. Academic Press, London, pp. 330–416.
Beadle, K., Singh, K.S., Troczka, B.J., Randall, E., Zaworra, M., Zimmer, C.T., Hayward, A., Reid, R., Kor, L., Kohler, M., Buer, B., Nelson, D.R., Williamson, M.S., Davies, T.G.E., Field, L.M., Nauen, R., Bass, C., 2019. Genomic insights into neonicotinoid sensitivity in the solitary bee *Osmia bicornis*. PLoS Genet. 15, e1007903.
Berenbaum, M.R., Johnson, R.M., 2015. Xenobiotic detoxification pathways in honey bees. Curr. Opin. Insect Sci. 10, 51–58.
Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bridges, A.R., Owen, M.D., 1984. The morphology of the honey bee (*Apis mellifera* L.) venom gland and reservoir. J. Morphol. 181, 69–86.
Brodie, B.B., Axelrod, J., Cooper, J.R., Goudette, L., LaDu, B.N., Mitoma, C., Udenfriend, S., 1955. Detoxification of drugs and other foreign compounds by liver microsomes. Science 121, 603–604.
Claudianos, C., Ranson, H., Johnson, R.M., Biswas, S., Schuler, M.A., Berenbaum, M.R., Feyereisen, R., Oakeshott, J.G., 2006. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honey bee. Insect Mol. Biol. 15, 615–636.
Dennis, E.A., 1991. Phospholipases. In: Methods in Enzymology. vol. 197 Academic Press, San Diego.
Dotimas, E.M., Hider, R.C., 1987. Honeybee venom. Bee World 68, 51–70.
Feyereisen, R., 1999. Insect P450s enzymes. Annu. Rev. Entomol. 44, 507–533.
Feyereisen, R., 2011. Arthropod CYPomes illustrate the tempo and mode in P450 evolution. Biochim. Biophys. Acta 1814, 19–28.
Feyereisen, R., 2012. Insect CYP genes and P450 enzymes. In: Gilbert, L.I. (Ed.), Insect Molecular Biology and Biochemistry. Elsevier and Academic Press, pp. 236–316.
Feyereisen, R., Baldridge, G.D., Famsworth, D.E., 1985. A rapid method for preparing insect Microsomes. Comp. Biochem. Physiol. 82B 559–363.
Fine, J.D., Mullin, C.A., 2017. Metabolism of *N*-methyl-2-pyrrolidone in honey bee adults and larvae: exploring age related differences in toxic effects. Environ. Sci. Technol. 51, 360–368.
Gilbert, M.D., Wilkinson, C.F., 1974. Microsomal oxidases in the honey bee, *Apis mellifera* (*L.*). Pest. Biochem. Physiol. 4, 55–66.
Gilbert, M.D., Wilkinson, C.F., 1975. An inhibitor of microsomal oxidation from gut tissues of the honey bee (*Apis mellifera*). Comp. Biochem. Physiol. 50B, 613–619.
Glaser, K.B., Jacobs, R.S., 1986. Molecular pharmacology of manoalide. Inactivation of bee venom phospholipase A2. Biochem. Pharmacol. 25, 449–453.
Gong, Y., Diao, Q., 2017. Current knowledge of detoxification mechanisms of xenobiotic in honey bees. Ecotoxicology 26, 1–12.
Guengerich, F.P., 2001. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. Chem. Res. Toxicol. 14, 611–650.
Guengerich, F.P., Martin, M.V., Sohl, C.D., Cheng, Q., 2009. Measurement of cytochrome P450 and NADPH-cytochrome P450 reductase. Nat. Protoc. 4, 1245–1251.
Habermann, E., 1972. Bee and wasp venoms. Science 177, 314–322.
Hardstone, M.C., Scott, J.G., 2010. Is *Apis mellifera* more sensitive to insecticides than other insects? Pest Manag. Sci. 66, 1171–1180.
Hodgson, E., Plapp Jr., F.W., 1970. Biochemical characteristics of insect microsomes. J. Agric. Food Chem. 18, 1048–1055.
Iwasa, T., Motoyama, N., Ambrose, J.T., Roe, R.M., 2004. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. Crop Prot. 23, 371 – 378.
Johnson, R.M., 2015. Honey bee toxicology. Annu. Rev. Entomol. 60, 415–434.
Johnson, R.M., Wen, Z., Schuler, M.A., Berenbaum, M.R., 2006. Mediation of pyrethroid insecticide toxicity to honey bees (Hymenoptera: Apidae) by cytochrome P450 monooxygenases. J. Econ. Entomol. 99, 1046–1050.
Johnson, R.M., Pollock, H.S., Berenbaum, M.R., 2009. Synergistic interaction between in-hive miticides in *Apis mellifera*. J. Econ. Entomol. 102, 474–479.
Lee, S.S., Scott, J.G., 1989. An improved method for preparation, stabilization, and storage of house fly (Diptera: Muscidae) microsomes. J. Econ. Entomol. 92, 1559–1563.
Li, X., Schuler, M.A., Berenbaum, M.R., 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu. Rev. Entomol. 52, 231–253.
Liu, N., Li, M., Gong, Y., Liu, F., Li, T., 2015. Cytochrome P450s – their expression, regulation, and role in insecticide resistance. Pestic. Biochem. Physiol. 120, 77–81.
Manjon, C., Troczka, B.J., Zaworra, M., Beadle, K., Randall, E., Hertlein, G., Singh, K.S., Zimmer, C.T., Homem, 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, 1137–1143.
Mao, W., Rupasinghe, S.G., Johnson, R.M., Zangerl, A.R., Schuler, M.A., Berenbaum, M.R., 2009. Quercetin metabolizing CYP6AS enzymes of the pollinator *Apis mellifera* (Hymenoptera: Apidae). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 154, 427–434.
Mao, W., Schuler, M.A., Berenbaum, M.R., 2011. CYP9Q-mediated detoxification of acaricides in the honey bee (*Apis mellifera*). Proc. Natl. Acad. Sci. U. S. A. 108, 12657–12662.
Nouvian, K., Reinhard, J., Giurfa, M., 2016. The defensive response of the honeybee *Apis mellifera*. J. Exp. Biol. 219, 2505–3517.
O'Brien, R.D., 1959. Activiation of thionophpsphates by liver microsomes. Nature 183, 121–122.
Omura, T., Sato, R., 1964. The carbon monoxide-binding pigment of liver microsomes. J. Biol. Chem. 239, 2379–2385.
Peiren, N., Vanrobaeys, F., de Graaf, D.C., Devreese, B., Van Beeumen, J., Jacobs, F.J., 2005. The protein composition of honeybee venom reconsidered by a proteomic approach. Biochim. Biophys. Acta 1752, 1–5.
Schmidt, J.O., 1982. Biochemistry of insect venoms. Annu. Rev. Entomol. 27, 339–368.
Scott, J.G., 1996. Preparation of microsomes from insects and purification of CYP6D1 from house flies. Methods Enzymol. 272, 287–292.
Smirle, M.J., 1993. The influence of colony population and brood rearing intensity on the activity of detoxifying enzymes in worker honey bees. Physiol. Entomol. 18, 420–424.
Smirle, M.J., Robinson, G.E., 1989. Behavioural status and detoxification enzyme activity are related in worker honey bees. J. Insect Behav. 2, 286–289.
Smirle, M.J., Winston, M.L., 1987. Intercolony variation in pesticide detoxification by the honey bee (Hymeoptera: Apidae). J. Econ. Entomol. 80, 5–8.
The Honeybee Genome Sequencing Consortium, 2006. Insight into social insects from the genome of the honeybee *Apis mellifera*. Nature 443, 931–949.
Todeschini, V., Panini, M., Chiesa, O., Puggioni, V., Anaclerio, M., Vinsova, H., Kamler, M., Titera, D., Mazzoni, E., 2017. In vitro study on the inhibition of enzymatic systems in Italian and Carniolan honey bees by piperonyl butoxide new derivatives. Bull. Insectology 70, 237–244.
Vidau, C., Diogon, M., Aufauvre, J., Fontbonne, R., Viguès, B., Brunet, J.L., Texier, C., Biron, D.G., Blot, N., El Alaoui, H., Belzunces, L.P., Delbac, F., 2011. Exposure to sublethal doses of fipronil and thiacloprid highly increases mortality of honeybee previously infected by *Nosema ceranae*. PLoS One 6, e21550.
Yu, S.J., Robinson, F.A., Nation, J.L., 1984. Detoxification capacity in the in the honey bee, *Apis mellifera* (*L.*). Pest. Biochem. Physiol. 11, 360–368.
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, 35–37.
Zimmer, C.T., Bass, C., Williamson, M.S., Kaussmann, M., Wölfel, K., Gutbrod, O., Nauen, R., 2014. Molecular and functional characterization of CYP6BQ23, a cytochrome P450 conferring resistance to pyrethroids in European populations of pollen beetle, *Meligethes aeneus*. Insect Biochem. Mol. Biol. 45, 18–29.