Biochemical profiling of functionally expressed CYP6P9 variants of the malaria vector *Anopheles funestus* with special reference to cytochrome b_5 and its role in pyrethroid and coumarin substrate metabolism

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ABSTRACT

Cytochrome P450 monooxygenases (P450s) are well studied enzymes catalyzing the oxidative metabolism of xenobiotics in insects including mosquitoes. Their duplication and upregulation in agricultural and public health pests such as anopheline mosquitoes often leads to an enhanced metabolism of insecticides which confers resistance. In the laboratory strain *Anopheles funestus* FUMOZ-R the duplicated P450s CYP6P9a and CYP6P9b are highly upregulated and proven to confer pyrethroid resistance. Microsomal P450 activity is regulated by NADPH cytochrome P450 oxidoreductase (CPR) required for electron transfer, whereas the modulatory role of cytochrome b_5 (CYB5) on insect P450 activity is less clear. In previous studies CYP6P9a and CYP6P9b were recombinantly expressed in tandem with *An. gambiae* CPR using *E. coli*-expression systems and CYB5 added to the reaction mix to enhance activity. However, the precise role of CYB5 on substrate turn-over when combined with CYP6P9a and CYP6P9b remains poorly investigated, thus one objective of our study was to address this knowledge gap. In contrast to the *CYP6P9* variants, the expression levels of both *CYB5* and *CPR* were not upregulated in the pyrethroid resistant FUMOZ-R strain when compared to the susceptible FANG strain, suggesting no immediate regulatory role of these genes in pyrethroid resistance in FUMOZ-R. Here, for the first time we recombinantly expressed *CYP6P9a* and *CYP6P9b* from *An. funestus* in a baculovirus expression system using High-5 insect cells. Co-expression of each enzyme with CPR from either *An. gambiae* or *An. funestus* did not reveal noteworthy differences in catalytic capacity. Whereas the co-expression of *An. funestus* CYB5 – tested at different multiplicity of infection (MOI) ratios – resulted in a significantly higher metabolization of coumarin substrates as measured by fluorescence assays. This was confirmed by Michaelis-Menten kinetics using the most active substrate, 7-benzyloxymethoxy-4-trifluoromethylcoumarin (BOMFC). We observed a similar increase in coumarin substrate turnover by adding human CYB5 to the reaction mix. Finally, we compared by UPLC-MS/MS analysis the depletion rate of deltamethrin and the formation of 4'OH-deltamethrin by recombinantly expressed CYP6P9a and CYP6P9b with and without CYB5 and detected no difference in the extent of deltamethrin metabolism. Our results suggest that co-expression (or addition) of CYB5 with CYP6P9 variants, recombinantly expressed in insect cells, can significantly enhance their metabolic capacity to oxidize coumarins, but not deltamethrin.

Keywords: Cytochrome P450, cytochrome b5, *Anopheles funestus*, CYP6P9, resistance, pyrethroid

1. Introduction

Cytochrome P450 monooxygenases (P450s, encoded by *CYP* genes) are a diverse superfamily of membrane-bound heme-thiolate enzymes described across all kingdoms of life (Nelson, 2018), and involved in the oxidation of a vast range of endogenous and exogenous substrates (Coon, 2005; Esteves et al., 2021; Feyereisen, 2012; Schuler, 2011). P450s play a key role in the metabolism of xenobiotics (Lu et al., 2021; Nauen et al., 2022), including insecticides in many pests of agricultural and public health importance including mosquito vectors of human diseases (Feyereisen, 2012; Vontas et al., 2020). The control of *Anopheline* malaria vectors over the last decades has heavily relied on pyrethroids such as deltamethrin, cypermethrin and permethrin, incorporated in insecticide treated bed nets (ITN) and applied as indoor residual sprays (IRS) (WHO, 2018). This chemical class of insecticides acts on voltage-gated sodium channels in the central nervous system and induces a quick knock-down of pest insects upon contact exposure (Soderlund, 2020). Due to frequent applications and continuous selection pressure, mosquitoes have developed resistance to pyrethroids that is often linked to the upregulation of P450 isoforms, which facilitate pyrethroid metabolism in resistant phenotypes (Hemingway and Ranson, 2000). In *Anopheles funestus* s.s., one of the major malaria transmitting mosquitoes in Sub-Saharan-Africa (Coetzee and Koekemoer, 2013), a number of P450s have been associated with pyrethroid resistance, including CYP6P9a, CYP6P9b, CYP6M7, CYP6AA1, CYP9J11, CYP6Z1 and, very recently, CYP325A (Ibrahim et al., 2016a, 2018; Riveron et al., 2013, 2014, 2017; Wamba et al., 2021). Indeed, *CYP6P9a* and *CYP6P9b* are highly upregulated in *An. funestus* and have been functionally shown to play a key role in the oxidative metabolism of pyrethroids (Cuamba et al., 2010; Ibrahim et al., 2015; Riveron et al., 2013; Weedall et al., 2019). This upregulation was first demonstrated in one of the global laboratory reference strains, FUMOZ-R (Wondji et al., 2009), originally collected in 2000 in Mozambique (Brooke et al., 2001), and since then maintained in the laboratory under pyrethroid selection pressure (Hunt et al., 2005).

Functional validation of the importance of upregulated P450 isoforms in conferring insecticide resistance in both agricultural pests and mosquitoes is usually provided by their recombinant expression and subsequent analysis of insecticide depletion and metabolite formation *in vitro*, or alternatively, by the ectopic expression of candidate genes in model insects such as *Drosophila melanogaster* (Nauen et al., 2022). The most important systems employed for the functional expression of insect P450s are based on *Escherichia coli* and insect cell lines utilizing a baculovirus expression system (reviewed in (Nauen et al., 2021). Interestingly the majority of mosquito P450s involved in insecticide resistance, including *An. funestus* CYP6P9a and CYP6P9b, have been expressed using *E. coli* along with *Anopheles gambiae* cytochrome P450 reductase (CPR) (Table 1). CPR is an essential membrane-bound flavoprotein, located in close vicinity to P450s, and the principal redox-partner of microsomal P450s required for electron transfer utilizing NADPH as a co-factor (Gutierrez

et al., 2003). In mammals, CPR has been shown to have many more essential functions, e.g., in steroid hormone synthesis, cholesterol homeostasis, heme catabolism and cholesterol biosynthesis (Porter, 2012). Its essential role is reflected by the fact that the germline deletion of CPR in mice was embryonic lethal (Shen et al., 2002). However, such studies are lacking in insects, but silencing of CPR by RNAi in *An. gambiae* showed enhanced sensitivity to permethrin (Lycett et al., 2006), while pest invertebrates resistant to insecticides resulted in increased insecticide sensitivity compared to susceptible individuals, indicating that lower expression of CPR negatively affected P450-mediated metabolism (Moural et al., 2020; Shi et al., 2015; Zhu et al., 2012).

The coupling of insect microsomal P450s and CPR in heterologous expression systems is essential for catalytic activity, whereas the role of another potential electron donor, cytochrome b_5 (CYB5), which is of particular importance in mammals (Schenkman and Jansson, 2003), remains nebulous in insects. Drug metabolism by several mammalian microsomal P450 isoforms was shown to be maximized by CYB5, either catalytically or allosterically, because its deletion resulted in a marked decrease in activity of several hepatic P450s (Finn et al., 2008; McLaughlin et al., 2010). CYB5 was also shown to significantly modulate the activity of some major human P450s such as CYP3A4 and CYP2D6 (Henderson et al., 2015). Therefore, CYB5 is considered to stimulate hepatic drug metabolism in combination with several P450 isoforms, rather than being an auxiliary player (Porter, 2012). However, despite its functional role, germline deletion of CYB5 in mice was not lethal, possibly indicating that other redox-proteins may substitute for its function (Finn et al., 2011). Its modulating role on insect P450 activity was first demonstrated using house fly microsomal preparations, where its inhibition resulted in decreased O-dealkylation of two coumarin substrates, while the metabolism of resorufins remained unaffected (Zhang and Scott, 1994). Another study confirmed its role as a modulator of house fly P450 activity by enhancing heptachlor epoxidation in a reconstituted system with CPR and CYP6A1 (Guzov et al., 1996). A more recent study revealed that CYB5 significantly increased the O-deethylation of 7-ethoxycoumarin by CYP6FD1 from *Locusta migratoria* (Liu et al., 2020). Although CYB5 was cloned and sequenced from two major anopheline mosquitoes, *An. gambiae* and *An. funestus*, many years ago (Matambo et al., 2010; Nikou et al., 2003), functional studies investigating its role in combination with CPR and pyrethroid-metabolizing P450s such as CYP6P9a and CYP6P9b are lacking.

To evaluate the role of CYB5 on the activity of recombinantly expressed insect P450s, it can either be co-expressed with the respective CPR and P450 of interest or added to the reaction mix, as done in most studies with heterologously expressed mosquito P450s utilizing an *E. coli* expression system (Table 1). *Aedes aegypti* CYP9M6 and CYP6BB2 were one of the few mosquito P450s heterologously expressed in Sf9 cells using the baculovirus system, but without CYB5 (Kasai et al., 2014). To the best of our knowledge only four *Anopheline* P450 isoforms, i.e. CYP6AA3 and CYP6P7 from *An. minimus* and

CYP6Z1 and CYP6Z2 from *An. gambiae,* were yet expressed using a baculovirus expression system (Boonsuepsakul et al., 2008; Duangkaew et al., 2011). Whereas CYP6AA3 and CYP6P7 were co-expressed with *An. minimus* CPR (without CYB5), *An. gambiae* CYP6Z1 and CYP6Z2 were co-expressed with *M. domestica* CPR and *D. melanogaster* CYB5 (Chiu et al., 2008). None of the pyrethroid-metabolizing *An. funestus* P450s have yet been expressed in insect cells using a baculovirus expression system. Interestingly, most, if not all, of the functional insecticide metabolization assays with recombinantly expressed *An. funestus* P450s, particularly CYP6P9a and CYP6P9b, relied on the co-expression of CPR from *An. gambiae* and the addition of *An. gambiae* CYB5 (Table 1, and references cited there-in).

The role of CYB5 on substrate turn-over when combined with CYP6P9a and CYP6P9b has not been fully investigated, so the objective of our study was to address this knowledge gap. In this study, we expressed for the first time *An. funestus* CYP6P9a and CYP6P9b in High-5 cells utilizing a baculovirus expression system. We compared the impact of co-expressed *An. gambiae* CPR and *An. funestus* CPR in a fluorescent probe assay on CYP6P9-mediated substrate conversion. Furthermore, we either co-expressed or added *An. funestus* CYB5 to reaction mixes with recombinantly expressed CYP6P9a and CYP6P9b (and CPR) and tested different MOIs (multiplicity of infection rates). Finally, we conducted metabolism studies with and without co-expressed CYB5 to evaluate its impact on deltamethrin metabolism catalyzed by CYP6P9a and CYP6P9b.

2. Materials and methods

2.1 Chemicals

Deltamethrin (CAS: 52918-63-5), β-Nicotinamide adenine dinucleotide 2'-phosphate (NADPH) reduced tetrasodium salt hydrate (CAS: 2646-71-1 anhydrous, purity ≥93 %), 7-ethoxycoumarin (EC; CAS: 31005-02-4, >99 %), 7-methoxy-4-trifluoromethylcoumarin (MFC; CAS: 575-04-2, ≥99 %), 7-Ethoxy-4-trifluoromethylcoumarin (EFC; CAS: 115453-82-2, ≥98 %) 7-benzyloxy-4-trifluoromethylcoumarin (BFC; CAS: 220001-53-6, ≥ 99 %), 7- hydroxy-coumarin (HC; CAS: 93-35-6, 99 %) 7-hydroxy-4-trifluoromethylcoumarin (HFC; CAS: 575-03-1, 98) were purchased from Sigma Aldrich/Merck (Darmstadt, Germany). 7-benzyloxymethoxy-4-trifluoromethylcoumarin (BOMFC; CAS: 277309-33-8; purity 95 %) was synthesized by Enamine Ltd. (Riga, Latvia). 7-pentoxycoumarin and 4'OH-deltamethrin (CAS: 66855-89-8) were internally synthetized (Leverkusen, Germany). Human CYB5 was purchased from Sigma (St. Louis, MO, USA; product no. C1427). All other chemicals and solvents were of analytical grade unless otherwise stated.

2.2 Insects

Anopheles funestus strains FANG and FUMOZ-R are known reference strains susceptible and resistant to pyrethroids (Amenya et al., 2008), respectively. Both strains were kept at 27.5 ± 0.5 °C, 65 ± 5 % relative humidity and a photoperiod of 12/12 L:D with one-hour dusk/dawn period, under laboratory conditions as described elsewhere (Nolden et al., 2021). The LC_{50}-values for deltamethrin against adults of strain FANG and FUMOZ-R maintained in our laboratory were 0.021 (CL95%: 0.015-0.027) and 4.61 (CL95%: 2.73-7.50) mg/m² in glazed tile bioassays, respectively, resulting in a resistance ratio of >200-fold (Nolden et al., 2021).

2.3 mRNA extraction and RT-qPCR

RNA was extracted from ten 3-5 days old adult females of strain FANG and FUMOZ-R TRIzol™ reaction kit following manufacturer's instructions. Afterwards RNA was purified using RNAeasy MINI Kit (Qiagen, Hilden, Germany) following manufacturer's instructions, including a DNAse-digest (RNase-free DNAse Set, 79254, Qiagen, Hilden, Germany) (modifications: Trizol incubation: 10 min, the column containing RNA sample was eluated twice to enhance RNA yields). RNA quantity was determined photometrically by measuring 260/280 nm and 230/260 nm ratios (NanoQuant Infinite 200, Tecan, Switzerland). All samples were adjusted to 20 ng/µL and RNA quality was checked using QIAxcel capillary electrophoresis as recently described (Nolden et al., 2021). For cDNA synthesis 0.3 µg of total RNA in 20 µL reaction volume was used employing IScript cDNA synthesis Kit (Bio-Rad, Hercules, USA).

Expression levels of the potential P450 redox partners CYB5 and CPR were measured by RT-qPCR following the method described earlier (Boaventura et al., 2020) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, USA) with a total volume of 10 µL using Real-Time CFX384™system (Bio-Rad, Hercules, USA). Samples were run in triplicate and a non-template control was included as negative control. Two µL of cDNA with 5 ng μL^{-1} of each primer with 200 nM final concentrations were used following the PCR program recently described (Nolden et al., 2021). Two reference genes were employed for normalization, *ribosomal protein S7* (*RPS 7*) and *actin 5c* (*Act*). Primer efficiencies were as follows: CYB5 99.5 % and CPR 100 %. The experiment was replicated (biological replicates) at least three times. Primer sequences and GenBank accession numbers of all relevant genes are given in Table S1.

2.4 Recombinant expression of CYP genes and its redox partners in insect cells

Gene sequences of *An. funestus CYP6P9a*, *CYP6P9b*, CPR (*AfCPR*), *CYB5*, and *An. gambiae* CPR (*AgCPR*) were retrieved from GenBank (Table S1). The respective expression plasmids were created using GeneArt server (Thermo Fisher), and PFastBac1 with BamHI and HindIII restriction sites was chosen. The sequences were codon optimized for final expression in High-Five cells (*Trichoplusia ni*). A PFastBac1 vector containing no insert served as a control. For the recombinant expression of P450

genes and their respective redox partners we followed the baculovirus expression protocol previously published (Manjon et al., 2018). In brief: MaxEfficiencyDH10 (Invitrogen, Waltham, MA, USA) competent *E. coli* cells containing a baculovirus shuttle vector (bacmid) were transformed according to manufacturer's instructions. The final bacmid was extracted using Large construct Kit (Qiagen, Hilden, Germany) following standard protocols. Subsequently Sf9 cells (Gibco™, kept in Sf-900-SFM (1X) cell culture medium, containing 25 µg/ml gentamycin) were virus transfected and the virus titer was determined according to Rapid Titer Kit (Takara Bio, San Jose, CA, USA).

High five cells were kept at 27 °C and 120 rpm in Express five medium (SFM (1X), Gibco™, Thermo Fisher, Waltham, MA, USA) containing 18 mM GlutaMAX (100X, Gibco™) and 10 µg mL^{-1} gentamycin (Gibco™). Preliminary experiments revealed highest CYP6P9a and CYP6P9b activity with a multiplicity of infection (MOI) of 1:0.5 for CYP6P9a/b:CPR (Figure S2). To obtain the best working MOI for CYB5 co-expression we tested the following MOIs (CYP6P9a/b:CPR:CYB5): 1:0.5:0.1; 1:0.5:0.2; 1:0.5:0.5. Cells were diluted to a concentration of $1.5x10^6$ cells mL^{-1} and incubated with 0.5 % fetal bovine serum (FBS; Sigma Aldrich), 0.2 mM delta-aminolevulinic acid (d-ALA; Sigma Aldrich), 0.2 mM Fe III citrate (Sigma Aldrich) and the respective amount of virus for 52 hours at 27 °C and 120 rpm. After harvesting, cells were resuspended in homogenization buffer (0.1 M K_2HPO_4, 1 mM DTT, 1mM EDTA, 200 mM saccharose, pH 7.6). FastPrep device (MP Biomedicals, Irvine, CA, USA) was used for grinding the cells following a 10 min centrifugation step at 4 °C and 700 g. The resulting supernatant was centrifuged for one hour at 100,000 g and 4 °C. The resulting microsomal pellet was resuspended with a Dounce tissue grinder in buffer (0.1 M K_2HPO_4, 0.1 mM EDTA, 1 mM DTT, 5 % Glycerol, pH 7.6) and protein amount was determined according to Bradford (Bradford, 1976). The functional expression of P450s was validated by their capacity to metabolize coumarin substrates and deltamethrin, and their concentrations were calculated based on CO difference spectra as described elsewhere (Omura and Sato, 1964).

2.5 Fluorescent probe bioassays

The enzymatic activity and substrate profile of each functionally expressed CYP6P9 isoform co-expressed with CPR (± CYB5) at different MOIs was measured in 384-well plates with six different coumarin substrates using the same fluorescent probe assay as recently described (Haas and Nauen, 2021; Nolden et al., 2021). *An. funestus* CYB5 was either co-expressed with the different CYP6P9 variants, or commercial human CYB5 was added to the reaction mix at a concentration of 0.8 µM. This concentration was based on other studies utilizing *An. gambiae* CYB5 (Table 1). Each assay was replicated four times. Michaelis Menten kinetics of BOMFC O-debenzylation by CYP6P9a and CYP6P9b with and without *An. funestus* CYB5 in order to check the impact of CYB5 on substrate conversion

followed the same protocol as mentioned above. All incubations were done under conditions linear with respect to time and protein concentration.

2.6 UPLC-MS/MS measurement of deltamethrin metabolism

UPLC-MS/MS analysis was carried out with slight modifications as previously described (Manjon et al., 2018). Briefly, for the chromatography on an Agilent 1290 Infinity II, a Waters Acquity HSS T3 column (2.1 x 50 mm, 1.8 mm) with 2 mM ammonium-acetate in methanol and 2mM ammonium-acetate in water as the eluent in gradient mode was employed. After positive electrospray ionization, ion transitions were recorded on a Sciex API6500 Triple Quad. Deltamethrin and 4'OH deltamethrin were measured in positive ion mode (ion transitions: deltamethrin 523.000 > 281.000, 4'OH deltamethrin 539.000 > 281.000). The peak integrals were calibrated externally against a standard calibration curve. The linear ranges for the quantification of deltamethrin and 4'OH deltamethrin were 0.5 - 100 ng/mL and 0.1 - 200 ng/mL, respectively. Samples were diluted prior to measurement if needed. The experiment was replicated thrice.

2.7 Data analysis

Gene expression analysis was done by employing Bio-Rad CFX Maestro 1.0 v 4.0 software (Bio-Rad, 2017, Hercules, USA) followed by subsequent unpaired t-tests in qbase (Biogazelle, Zwijnaarde, Belgium) to compare for significant differences in gene expression levels. Michaelis Menten kinetics were analyzed by nonlinear regression using Graph Pad Prism 9.0 (GraphPad Software Inc., CA, USA). CYB5 sequence alignments were conducted using the Geneious Alignment tool in Geneious software v. 10.2.3 (Biomatters Ltd., New Zealand).

3. Results

3.1 Expression levels of CPR and CYB5

As described previously LC_{50}-values for deltamethrin against adults of *An. funestus* strain FANG and FUMOZ-R maintained in our laboratory were 0.021 (CL95%: 0.015-0.027) and 4.61 (CL95%: 2.73-7.50) mg/m² in glazed tile bioassays, respectively, resulting in a resistance ratio of >200-fold (Nolden et al., 2021). Deltamethrin resistance in strain FUMOZ-R is correlated with the upregulation of *CYP6P9a* and *CYP6P9b* in comparison to strain FANG (Figure 1a). In contrast to the *CYP6P9* variants, the expression levels of both *CYB5* and *CPR* as measured by RT-qPCR were not upregulated in female adults of the pyrethroid resistant FUMOZ-R strain when compared to the susceptible FANG strain, suggesting no immediate regulatory role of these P450 redox partners in pyrethroid resistance in FUMOZ-R (Figure 1b).

3.2 Functional expression and coumarin substrate profiling of An. funestus CYP6P9 variants in concert with CPR and CYB5 in insect cells

The heterologous baculovirus-mediated expression of CYP6P9a and CYP6P9b in High-5 cells co-infected with *An. gambiae* CPR (AgCPR) at different multiplicity of infection (MOI) ratios, revealed highest fluorescent probe substrate metabolization capacity in the presence of NADPH at a P450:CPR ratio of 1:0.5 across six different alkylated and benzylated coumarins (Table S2). No basal metabolizing activity against any of the six coumarin probe substrates was detected when microsomal membranes resulting from mock virus infections were incubated with NADPH (data not shown), suggesting that the observed substrate profile is based on the expression of the respective *An. funestus* CYP6P9 variant. This confirmed functional expression in High-5 cells despite weak CO difference spectra (Figure S2). The O-debenzylation of 7-benzyloxymethoxy-4-(trifluoromethyl)-coumarin (BOMFC) resulting in 7-hydroxy-4-(trifluoromethyl)coumarin (HC) revealed the highest enzyme activity with both P450s at all tested P450:CPR MOIs (Figure 3A-C), followed by the O-debenzylation of 7-benzyloxy-4-trifluoromethylcoumarin (BFC). Less preferred substrates were the alkylated coumarin derivatives tested such 7-methoxy-4-trifluoromethylcoumarin (MFC). However, we observed a slight, but significant difference between CYP6P9a and CYP6P9b in their ability to metabolize BFC and EFC, with CYP6P9b showing higher activity. The overall results and trends in coumarin substrate preference did not change when both CYP6P9 isoforms were co-expressed with *An. funestus* CPR (AfCPR) instead of AgCPR at a MOI of 1:0.5 (Figure 2A and Figure S3D). Based on these results we decided to conduct all other experiments with recombinantly expressed CYP6P9a and CYP6P9b in concert with AfCPR.

Next, we tested the impact of the co-expression of *An. funestus* CYB5 (AfCYB5) on coumarin substrate metabolism at different MOIs in combination with CYP6P9 variants and AfCPR. The highest enzyme activity was obtained from microsomal preparations of High-5 cells infected at a MOI of 1:0.5:0.1 (P450:CPR:CYB5) (Figure 2B). Increasing the level of co-expressed AfCYB5 resulted in a significantly lower enzyme activity with all tested coumarin substrates (Figure 2B). The overall coumarin substrate profile of both CYP6P9 variants did not change when co-expressed with AfCYB5. However, at a MOI of 1:0.5:0.1 (P450:CPR:CYB5) the activity of both CYP6P9 isoforms was significantly higher with the preferred probe substrates BOMFC, BFC, and EFC when compared to CYP6P9 expressions without AfCYB5. Finally, we checked if the addition of a mammalian CYB5, commercial human CYB5, results in a similar increase in activity. We demonstrated that human CYB5 added to the reaction mix at 0.8 µM increased the activity of CYP6P9a and CYP6P9b co-expressed with AfCPR (MOI 1:0.5) without changing the substrate profile (Figure 2C), confirming its ability to substitute *An. funestus* or *An. gambiae* CYB5. The metabolic activity of both CYP6P9 variants towards preferred coumarin substrates is not

influenced by the choice of the CPR source (AgCPR vs. AfCPR), but the addition of CYB5 increased their activity up to ~ 6-fold depending on the coumarin substrate (e.g., BFC, Table S2).

3.3 Michaelis Menten kinetics of the O-debenzylation of BOMFC by CYP6P9 variants

Based on the probe substrate activity profiling presented above we have chosen the coumarin substrate BOMFC for a more detailed steady-state kinetic analysis with recombinantly expressed CYP6P9a and CYP6P9b with and without the co-expression of *An. funestus* CYB5. The rate of the O-debenzylation of BOMFC by recombinantly expressed CYP6P9a and CYP6P9b was time dependent and followed Michaelis-Menten kinetics in response to BOMFC concentration (Figure 3), resulting in a K_m-value of 4.07 µM (CI95%: 3.44-4.80) and 2.13 µM (CI95%: 1.62-2.79), and a catalytic activity K_{cat} of 1.59 ± 0.034 min^{-1} and 1.10 ± 0.034 min^{-1}, respectively. The co-expression of CYB5 did not significantly change the K_m-value obtained for BOMFC; CYP6P9a: 4.89 µM (CI95%: 4.10-5.83), and CYP6P9b: 3.25 µM (CI95%: 2.61-4.05). Whereas K_{cat} increased significantly at 2.83 ± 0.067 min^{-1} and 2.29 ± 0.064 min^{-1} in the presence of CYB5 for CYP6P9a and CYP6P9b, respectively. Thus, suggesting a supportive role of CYB5 in CYP6P9 driven BOMFC metabolism, and overall, a slightly lower catalytic capacity of CYP6P9a compared to CYP6P9b.

3.4 Deltamethrin metabolism by CYP6P9 variants with and without CYB5

UPLC-MS/MS analysis of microsomal preparations of High-5 cells expressing CYP6P9a and CYP6P9b, respectively, in concert with *An. funestus* CPR at a MOI of 1:0.5 revealed a time-dependent depletion of deltamethrin in the presence of NADPH (Figure 4A and 4B). The co-expression of *An. funestus* CYB5 did not change the metabolic efficiency of both enzymes. Furthermore, we confirmed the formation of 4'OH deltamethrin by both enzymes (Figure 4C and 4D), suggesting that the deltamethrin depletion is largely based on its metabolism rather than sequestration. The co-expression of CYB5 had no impact on the extent of hydroxylated deltamethrin formation. However, we noticed a stochiometric inconsistency between deltamethrin depletion and 4´OH deltamethrin formation, possibly suggesting the presence of other undetected metabolites.

4. Discussion

Metabolic resistance towards pyrethroids in Anopheline mosquitoes such as *An. funestus* is largely driven by upregulated P450s of which a number have been recombinantly expressed, most of them in bacterial expression systems using competent *E. coli* cells (Nauen et al., 2021; Vontas et al., 2020). Among upregulated P450s mediating pyrethroid resistance in *An. funestus,* CYP6P9a and CYP6P9b, were most prominent and shown to metabolize different pyrethroids when functionally expressed in

E. coli (Riveron et al., 2013; Weedall et al., 2019). Here we successfully expressed both P450 genes in combination with either AfCPR or AgCPR at different MOI ratios in High-5 cells employing a baculovirus expression system. Microsomal insect cell preparations revealed highest CYP6P9a/b activities with the fluorescent probe substrate BOMFC at a MOI ratio of 1:0.5 (P450:CPR), resembling findings with other insect P450s utilizing similar expression conditions (Bass et al., 2013; Manjon et al., 2018; Zimmer et al., 2018). Many functional studies conducted with recombinantly expressed *An. funestus* P450s showed that their co-expression with *An. gambiae* CPR as a surrogate for the homologous *An. funestus* CPR worked well (Table 1). Our study demonstrated and confirmed that the overall enzymatic activity of the duplicated CYP6P9 isoforms did not differ if co-expressed with either *An. gambiae* CPR or *An. funestus* CPR. Indeed, CPR incompatibilities as observed for *Tetranychus urticae* CYP392A11 and CYP392A16 ectopically expressed in phylogenetically distant *Drosophila melanogaster*, are rather unlikely in closely related species (Riga et al., 2020).

The baculovirus system recruiting High-5 cells for P450 expression is thought to offer some advantages over *E. coli* such as insect-specific posttranslational modifications of the expressed P450s, though meaningful comparative studies with insect P450s are lacking. Comparative studies utilizing prokaryotic and eukaryotic expression systems were especially conducted with human P450 isoforms (Hiratsuka, 2012), and revealed for example significantly different catalytic efficiencies in benzo[a]pyrene detoxification by functionally expressed human CYP1A1, possibly linked to differences in lipid membrane composition (Stiborová et al., 2017). Although optimized *E. coli* expression systems often resulted in higher P450 yields as demonstrated for human CYP3A4 and CYP17A1 (Schroer et al., 2010), it was shown that they have limitations in contrast to insect and mammalian cells (Kumondai et al., 2020). On the other hand, *E. coli* preparations do not express basal P450 activity as they lack endogenous P450s possibly interfering with those P450s recombinantly expressed (Nauen et al., 2021), which facilitates the screening of compounds for metabolic liabilities including insecticides (Lees et al., 2020; Yunta et al., 2019). Future studies with insect P450s are necessary to shed light on possible differences in P450 catalytic efficiency towards various substrates between prokaryotic and eukaryotic expression systems.

We recently confirmed high expression levels of the duplicated *CYP6P9* genes in the laboratory reference strain FUMOZ-R(Nolden et al., 2021), whereas *CPR*, the principal redox partner of microsomal P450s, is not overexpressed when compared to the susceptible strain FANG. Another potential redox partner, *CYB5*, often added or co-expressed along with mosquito P450s such as CYP6P9a and CYP6P9b (Table 1), is also not overexpressed in strain FUMOZ-R compared to FANG. In contrast to our findings, Nikou et al. (2003) found a 2.3-fold upregulation of *CYB5* expression in the pyrethroid resistant *An. gambiae* RSP strain in comparison to the susceptible Kisumu strain. This was

366 also demonstrated in a cypermethrin resistant strain of *Plutella xylostella* showing elevated *CPR* and
367 *CYB5* expression levels (Chen and Zhang, 2015). It would be interesting to evaluate *CPR* and *CYB5*
368 expression levels in pyrethroid resistant field-collected populations of *An. funestus* to rule out possible
369 effects related to the fact that FUMOZ-R has been kept without selection pressure under laboratory
370 conditions for many years. However, our qPCR data do not suggest an obvious role of CYB5 in the
371 amplification of CYP6P9-mediated pyrethroid resistance in *An. funestus* strain FUMOZ-R. This is
372 supported by experimental data showing no difference in deltamethrin depletion measured by UPLC-
373 MS/MS when recombinantly expressed CYP6P9a and CYP6P9b were co-expressed with and without
374 *An. funestus* CYB5. Interestingly, pyrethroid-mimetic activity-based probes were far less active in
375 labelling P450s in CYB5 deficient mouse microsomes, indicating potential species and/or enzyme
376 differences in CYB5 effects (Ismail et al., 2013). In contrast we were able to demonstrate that *An.*
377 *funestus* CYB5 co-expressed at a MOI ratio of 1:0.5:0.1 (P450:CPR:CYB5) significantly increased the
378 catalytic capacity of both CYP6P9a and CYP6P9b to O-debenzylate BOMFC and BFC, the preferred
379 coumarin substrates as recently shown with microsomal preparations of female adults of FUMOZ-R
380 (Nolden et al., 2021). Thus, suggesting that at least for some of the coumarin substrates CYB5 possibly
381 improved the electron transfer through CPR, but didn´t change the coumarin substrate profile.
382 However, the coumarin profiling results obtained in this study also indicated that both CYP6P9
383 isoforms contributed greatly to the microsomal P450 activity of the pyrethroid resistant FUMOZ-R
384 recently described (Nolden et al., 2021), and that CYB5 is not necessary to resemble *in vivo* microsomal
385 activity. Other tested MOI ratios using higher amounts of CYB5 resulted in lower enzyme activity,
386 possibly linked to lower overall expression yields of the respective CYP genes as for example shown for
387 human CYP1A2, CYP2C9 and CYP3A4 in other heterologous expression systems (Kumondai et al.,
388 2020). Studies with recombinantly expressed human CYP2B4 revealed that depending on the
389 P450:CYB5 ratio its catalytic efficiency was impaired, because CPR and CYB5 are competing for the
390 same binding site at the enzyme (Zhang et al., 2008). These findings are in line with our results of
391 different MOI ratios tested: A ratio of 1:0.5:0.1 (CYP:CPR:CYB5) revealed highest coumarin substrate
392 activity, whereas a ratio of 1:0.5:0.2 drastically reduced P450 activity.

393 CYP6M2 from *An. gambiae* was shown to better metabolize deltamethrin and permethrin if 0.8 µM
394 *An. gambiae* CYB5 was supplemented to the reaction (Stevenson et al., 2011), whereas in our study
395 with *An. funestus* CYP6P9 variants the co-expression of CYB5 did not enhance deltamethrin
396 metabolism, suggesting a minor, if any role for CYB5 in facilitating deltamethrin metabolism. Future
397 studies with CYP6P9a/b are necessary to clarify if this is also true for other pyrethroids than
398 deltamethrin. High-5 cell microsomal membranes are highly likely to contain endogenous CYB5
399 possibly sufficient to support P450 mediated deltamethrin hydroxylation as an additional redox
400 partner. Knock-out of the endogenous *CYB5* in High-5 cells by genome editing would help to address

this point. Such an endogenous CYB5 supply is absent in *E. coli* expression systems, possibly explaining why supplementation with exogenous *An. gambiae* CYB5 may have a larger impact, e.g., on recombinantly expressed CYP6M2 (Stevenson et al., 2011).

Quite a few studies demonstrated elevated microsomal levels of CYB5 linked to a resistant phenotype in insects. In a multiple resistant house fly strain microsomal P450 and CYB5 levels were found upregulated in some tissues (Zhang et al., 1998). Similar results were obtained with microsomal fractions of a carbamate and pyrethroid resistant *Blattella germanica* strain (Valles and Yu, 1996). In the cypermethrin resistant house fly LPR strain CYP6D1 and CYB5 were found to be upregulated, and inhibition of CYB5 with a specific antibody prevented the formation of 4'OH-cypermethrin (Liu and Scott, 1996; Zhang and Scott, 1996). In a previous study the same authors reported that specific CYB5 inhibition in house fly microsomes did not affect methoxyresorufin-O-demethylase and ethoxyresorufin-O-deethylase activity, but ethoxycoumarin-O-deethylase activity. Another study demonstrated an enhanced metabolism of heptachlor and aldrin if CYP6A2 from *D. melanogaster* was supplemented with CYB5 (Dunkov et al., 1997). This was also observed with recombinantly expressed house fly CYP6A1 and it was shown that even apo-CYB5 (devoid of heme) enhanced the metabolic activity against heptachlor and steroid-substrates, and it was concluded that CYB5 mainly enhanced metabolism by CYP6A1 in two ways, by delivering the second electron to CYP6A1 and by allosteric interactions (Murataliev et al., 2008). Similar interactions were also described between a number of recombinantly expressed human P450s and apo-CYB5 (Yamazaki et al., 2002). For recombinantly expressed CYP6FD1 from *L. migratoria* it was shown that silencing of the respective CYB5 gene, did not alter metabolism towards deltamethrin, chlorpyrifos, imidacloprid and carbaryl, but the co-expression of recombinant CYP6FD1 with CYB5 enhanced the metabolism of 7-ethoxycoumarin (Liu et al., 2020).

The examples above and our data obtained with CYP6P9 variants suggest that the involvement of CYB5 in xenobiotic P450-mediated metabolism depends on P450 and substrate-specificity, as reviewed earlier (Schenkman and Jansson, 2003). Our results suggest that co-expression (or addition) of CYB5 with CYP6P9 variants, recombinantly expressed in insect cells, can significantly enhance their metabolic capacity to degrade coumarins, but not deltamethrin.

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Declaration of competing interests

RN is employed by Bayer AG, a manufacturer of pesticides. MN is a PhD student affiliated with the LSTM and funded by the Innovative Vector Control Consortium (IVCC) and Bayer AG.

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697 **Table 1.** Selected examples of addition/co-expression of cytochrome b5 (CYB5) in heterologously expressed mosquito CYP genes

Insect	*CYP*	Expression system	CPR* origin	CYB5 origin	CYB5 addition	Reference
Ae. aegypti	*CYP6Z8*	Yeast	*Ae. aegypti*	*Ae. aegypti*	Ratio 80 pmol:50 pmol (b5:P450) added to reaction mix	(Chandor-Proust et al., 2013)
Ae. aegypti	*CYP6CB1*	*E. coli*	*An. gambiae*	*An. gambiae*	Ratio 0.8:0.1 µM (b5:P450) added to reaction mix	(Stevenson et al., 2012)
Ae. aegypti	*CYP9J19*	*E. coli*	*An. gambiae*	*An. gambiae*	Ratio 0.8:0.1 µM (b5:P450) added to reaction mix	Ibid.
Ae. aegypti	*CYP9J24*	*E. coli*	*An. gambiae*	*An. gambiae*	Ratio 0.8:0.1 µM (b5:P450) added to reaction mix	Ibid.
Ae. aegypti	*CYP9J26*	*E. coli*	*An. gambiae*	*An. gambiae*	Ratio 0.8:0.1 µM (b5:P450) added to reaction mix	Ibid.
Ae. aegypti	*CYP9J28*	*E. coli*	*An. gambiae*	*An. gambiae*	Ratio 0.8:0.1 µM (b5:P450) added to reaction mix	Ibid.
Ae. aegypti	*CYP9J32*	*E. coli*	*An. gambiae*	*An. gambiae*	Ratio 0.8:0.1 µM (b5:P450) added to reaction mix	Ibid.
Ae. aegypti	*CYP9M6*	bac-to-bac, Sf9	*Ae. aegypti*	*Ae. aegypti*	Not indicated	(Kasai et al., 2014)
Ae. aegypti	*CYP6BB2*	bac-to-bac, Sf9	*Ae. aegypti*	*Ae. aegypti*	Not indicated	Ibid.
An. arabiensis	*CYP6P4*	*E. coli*	*An. gambiae*	*An. gambiae*	Not indicated	(Ibrahim et al., 2016b)
An. funestus	*CYP6P9a/b*	*E. coli*	*An. gambiae*	*An. gambiae*	Ratio 0.8 µM:45 pmol (b5:P450) added to reaction mix	(Riveron et al., 2014)
An. funestus	*CYP6M7*	*E. coli*	*An. gambiae*	*An. gambiae*	Ratio 0.8 µM:45 pmol (b5:P450) added to reaction mix	Ibid.
An. funestus	*CYP6Z1*	*E. coli*	*An. gambiae*	*An. gambiae*	Not indicated	(Ibrahim et al., 2016a)
An. funestus	*CYP6AA1*	*E. coli*	*An. gambiae*	*An. gambiae*	Not indicated	(Ibrahim et al., 2018)
An. funestus	*CYP9J11*	*E. coli*	*An. gambiae*	*An. gambiae*	Ratio 0.8 µM:45 pmol (b5:P450) added to reaction mix	(Riveron et al., 2017)
An. gambiae	*CYP6M2*	*E. coli*	*An. gambiae*	*An. gambiae*	Ratio 0.8 µM:0.1 µM (b5:P450) added to reaction mix	(Stevenson et al., 2011)
An. gambiae	*CYP6P3*	*E. coli*	*An. gambiae*	-	Without *CYB5*	(Müller et al., 2008)
An. gambiae	*CYP6Z2*	*E. coli*	*An. gambiae*	-	Without *CYB5*	(Mclaughlin et al., 2008)
An. gambiae	*CYP6Z1/2*	bac-to-bac, Sf9	*M. domestica*	*D. melanogaster*	co-expression, MOI 2:2:0.1	(Chiu et al., 2008)
An. gambiae	*CYP9J5*	*E. coli*	*An. gambiae*	*An. gambiae*	Ratio 10:1 (b5:P450) added to reaction mix	(Yunta et al., 2016) (2019)
An. gambiae	*CYP6P4*	*E. coli*	*An. gambiae*	*An. gambiae*	Ratio 10:1 (b5:P450) added to reaction mix	Ibid.
An. gambiae	*CYP6P2*	*E. coli*	*An. gambiae*	*An. gambiae*	Ratio 10:1 (b5:P450) added to reaction mix	Ibid.
An. gambiae	*CYP6P5*	*E. coli*	*An. gambiae*	*An. gambiae*	Ratio 10:1 (b5:P450) added to reaction mix	Ibid.
An. minimus	*CYP6AA3*	bac-to-bac, Sf9	*An. minimus*	-	Without *CYB5*	(Boonsuepsakul et al., 2008)
An. minimus	*CYP6P7*	bac-to-bac, Sf9	*An. minimus*	-	Without *CYB5*	(Duangkaew et al., 2011)

698 * CPR = cytochrome P450 reductase

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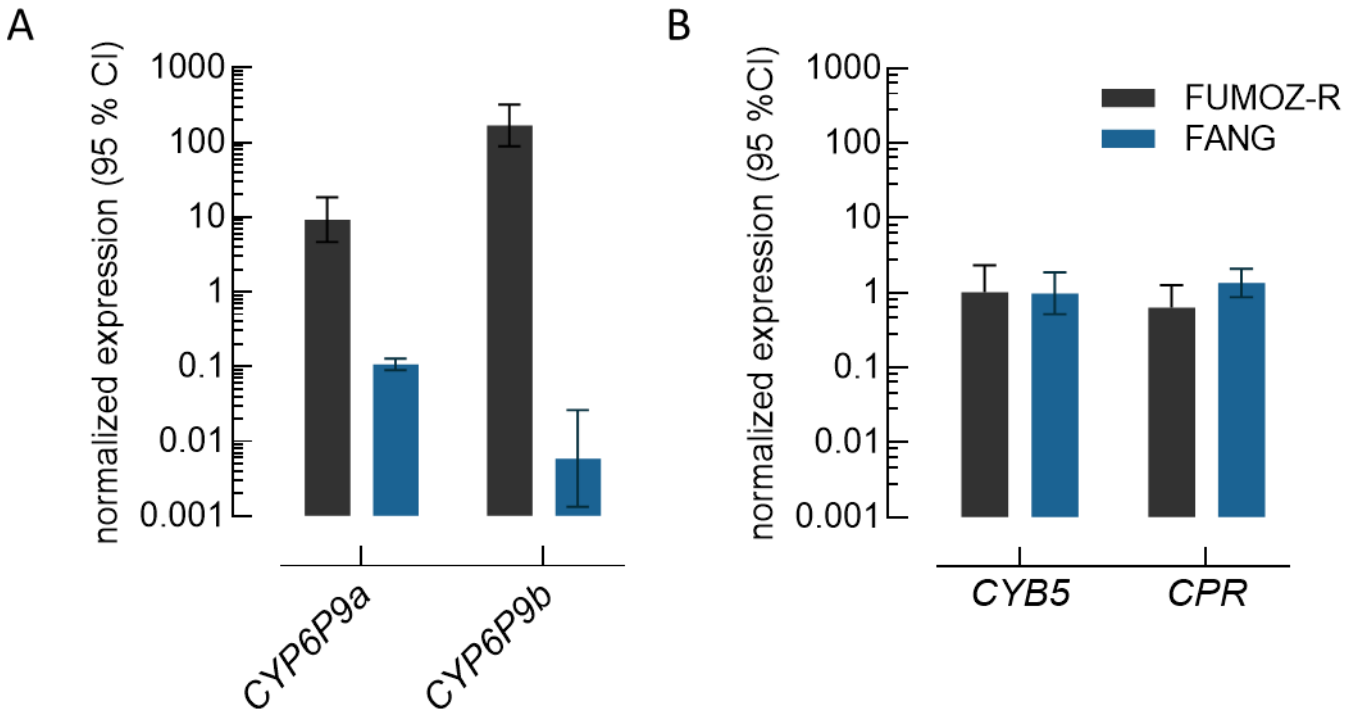

Figure 1. **RT-qPCR analysis of gene expression in female adults.** Expression level of (A) *CYP6P9a* and *CYP6P9b*, and (B) cytochrome b5 (CYB5) and cytochrome P450-reductase (CPR) of *An. funestus* strains FUMOZ-R and FANG measured by RT-qPCR. The expression levels were normalized to *RPS7* and *Act* (5c) reference genes. Data are mean values ± 95% CI (n=4). The expression data shown in (A) were taken from Nolden et al. (2021).

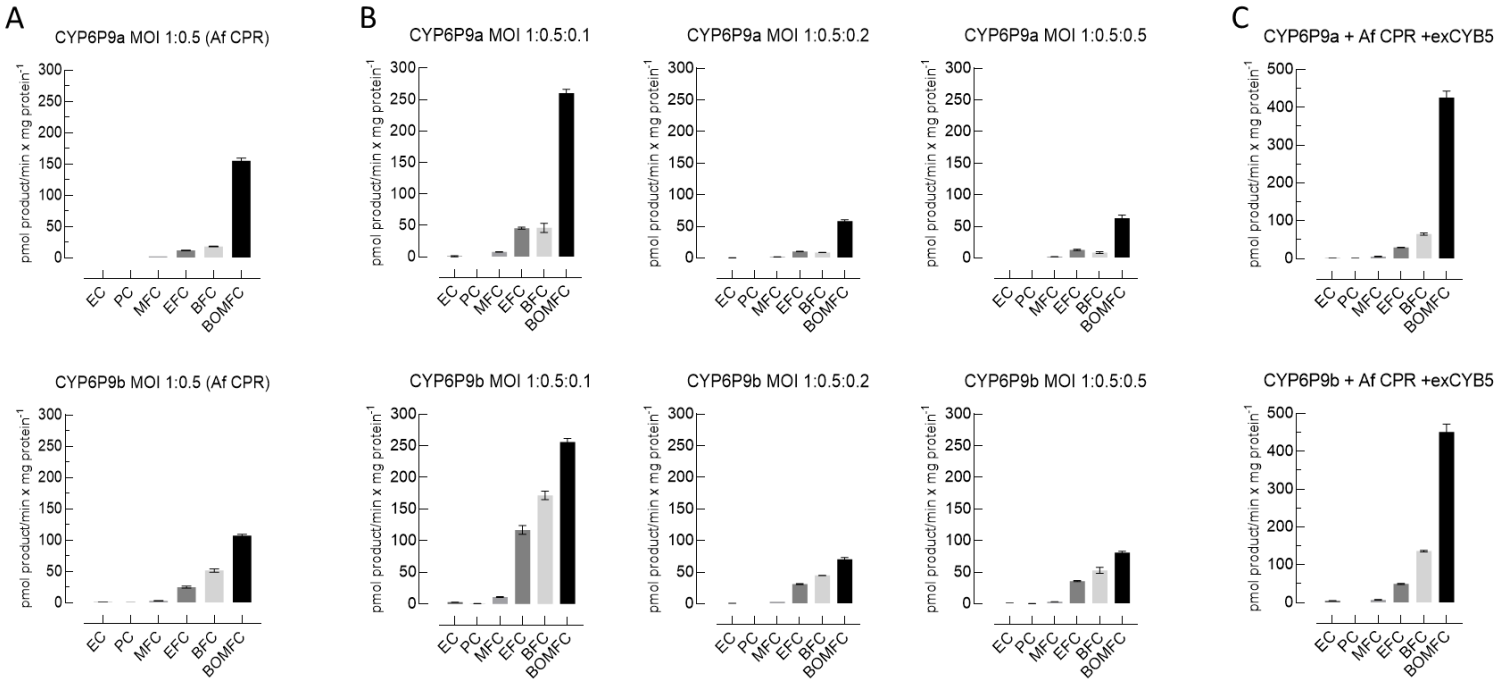

Figure 2. **Coumarin substrate profiling of recombinantly expressed CYP6P9a and CYP6P9b co-expressed with *An. funestus* CPR with and without CYB5.** (A) Coumarin substrate metabolism by recombinantly expressed CYP6P9a and CYP6P9b co-expressed with *An. funestus* cytochrome P450-reductase (Af CPR) at MOI 1:0.5, (B) co-expressed with *An. funestus* CYB5 at different MOI ratios stated as P450:CPR:CYB5, and (C) supplemented with exogenous 0.8 µM human CYB5 (exCYB5) while expressed at MOI 1:0.5 (P450:CPR). Data are mean values ± SD (n=4). Abbreviations: BFC, 7-benzyloxy-4-trifluoromethyl coumarin; MFC, 7-methoxy-4-trifluoromethyl coumarin; EFC, 7-ethoxy-4-rifluoromethyl coumarin; BOMFC, 7-benzyloxymethoxy-4-trifluoromethyl coumarin; PC, 7-n-pentoxy coumarin; EC, 7-ethoxy coumarin.

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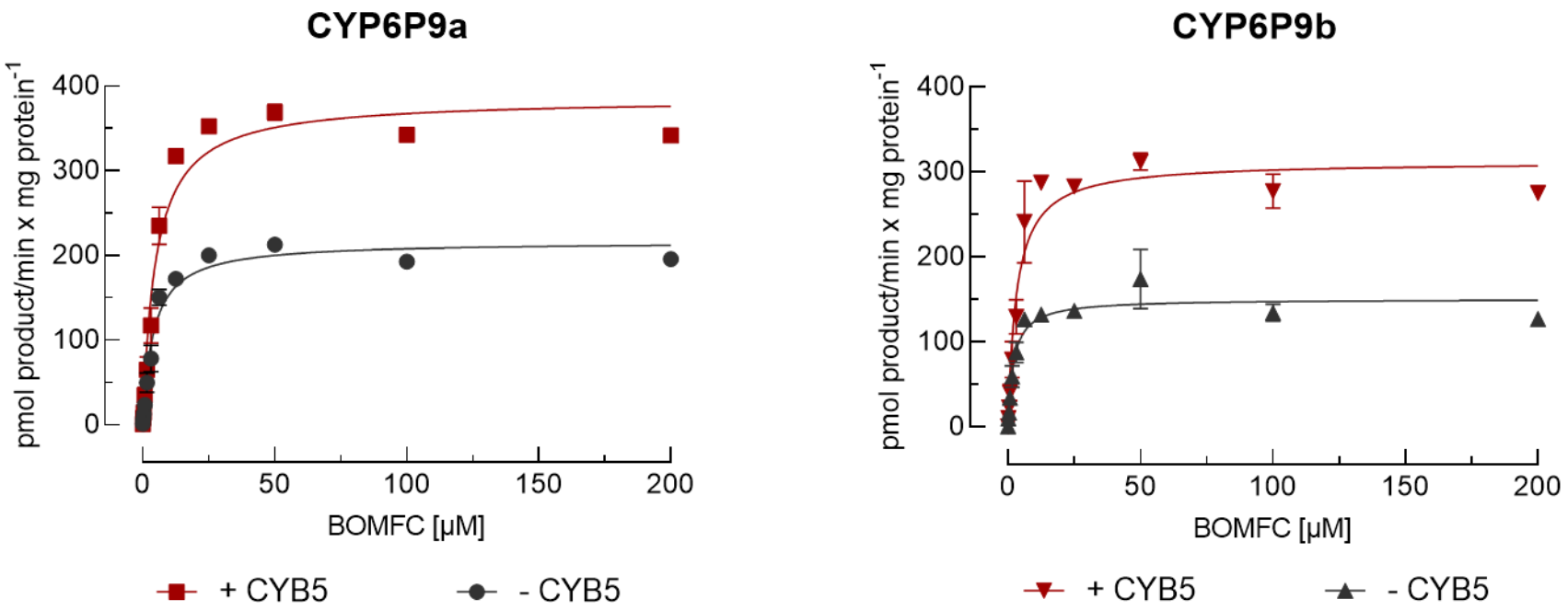

Figure 3. Steady-state enzyme kinetics of product formation. Michaelis-Menten kinetics of BOMFC O-debenzylation leading to 7-hydroxy-4-(trifluoromethyl)coumarin (HC) by recombinantly expressed CYP6P9a and CYP6P9b (co-expressed with AfCPR, MOI 1:0.5) with and without co-expression of cytochrome b5 (CYB5; MOI 1:0.5:0.1). Data are mean values ± SD (n=4). K_m- and V_{max}-values (and 95% confidence intervals) were calculated by nonlinear regression analysis using GraphPad Prism 9.0.

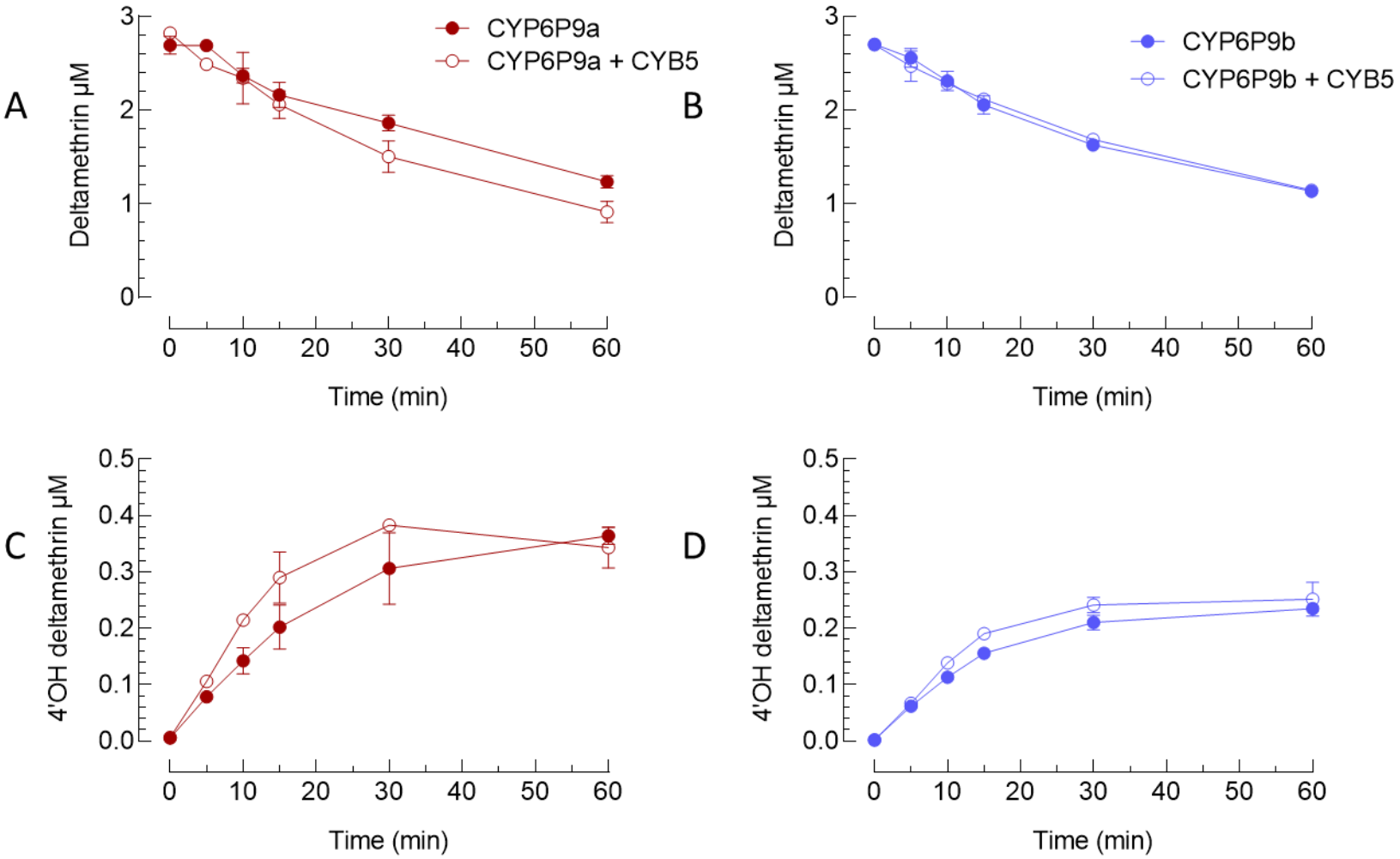

Figure 4. **Kinetics of deltamethrin metabolism by baculovirus-expressed *An. funestus* CYP6P9 variants.** Deltamethrin depletion and formation of the respective 4'OH metabolite in the presence of NADPH by recombinantly expressed *An. funestus* CYP6P9a (A,C) and CYP6P9b (B,D) co-expressed with (open circles) and without (closed circles) *An. funestus* cytochrome b5 (CYB5). Data are mean values ± SD (n=3).