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Isolation of the monooxygenase complex from *Rhipicephalus (Boophilus) microplus* – clues to understanding acaricide resistance

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Abstract

The monooxygenase complex is composed of three key proteins, a cytochrome P450 (CYP), the cytochrome P450 oxidoreductase (CPR) and cytochrome b_5 and plays a key role in the metabolism and detoxification of xenobiotic substances, including pesticides. In addition, overexpression of these components has been linked to pesticide resistance in several important vectors of disease. Despite this, the monooxygenase complex has not been isolated from the Southern cattle tick *Rhipicephalus* (*Boophilus*) *microplus,* a major disease vector in livestock.

Using bioinformatics 115 transcriptomic sequences were analyzed to identify putative pesticide metabolizing CYPs. RACE-PCR was used to amplify the full length sequence of one CYP; CYP3006G8 which displays a high degree of homology to members of the CYP6 and 9 subfamilies, known to metabolize pyrethroids. mRNA expression levels of CYP3006G8 were investigated in 11 strains of *R. microplus* with differing resistance profiles by qPCR, the results of which indicated a correlation with pyrethroid metabolic resistance. In addition to this gene, the sequences for CPR and cytochrome b_5 were also identified and subsequently isolated from *R. microplus* using PCR.

CYP3006G8 is only the third CYP gene isolated from *R. microplus* and the first to putatively metabolize pesticides. The initial results of expression analysis suggest that CYP3006G8 metabolizes pyrethroids but further biochemical characterization is required to confirm this. Differences in the kinetic parameters of human and mosquito CPR in terms of NADPH binding have been demonstrated and could potentially be used to design species specific pesticides. Similar differences in the tick CPR would confirm that this is a characteristic of heamatophagous arthropods.

Keywords: Cytochrome P450 (CYP); cytochrome P450 oxidoreductase (CPR); cytochrome b_5; *Rhipicephalus microplus*; pesticide resistance.

Introduction

The detoxification of xenobiotics is an essential function for life and is carried out by three specific enzyme families: Glutathione S transferases, Esterases (or Carboxylesterases) and the Cytochrome P450s (CYPs). Cytochrome P450s are an ancient protein family found in all forms of life (Scott and Wen 2001; Sztal et al., 2012) and are known to be the major phase I metabolizing enzymes. In humans, CYPs metabolize approximately 65% of currently used clinical drugs (Zhou et al., 2005), emphasising the importance of this group of enzymes in xenobiotic metabolism. CYPs are characterized by the presence of a highly conserved heme-binding domain located towards the C terminus of the protein. This signature motif, with the sequence FxxGxxxCxG is found in all CYPs (Syed and Mashele 2014; Werck-Reichhart and Feyereisen 2000) and plays a vital role in the electron transport mechanism used by these enzymes to, in many cases, incorporate molecular oxygen into xenobiotics thus rendering them more water soluble and more readily excreted from the cell (Mamidala et al., 2011).

Xenobiotic metabolism is an important topic of investigation amongst arthropods due to this group of organisms containing numerous vectors of medical/veterinary diseases and a host of agricultural pests that are developing resistance to commonly used chemical control agents (Brogdon and McAllister 1998). Amongst the arthropods, there has been a huge amount of research into species such as *Musca domestica* (house fly) and *Drosophila melanogaster* (fruit fly) due to their use as model organisms and of course various mosquito

species due to the medical and economic importance (Drali et al., 2012; Kawada et al., 2011; Kushwah et al., 2015; Wondji et al., 2011).

Pesticide resistance is not a novel problem but one that is ever increasing due to its association with disease transmission to both animals and plants. Pesticide resistance has been described by the World Health Organisation (WHO) as “the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject” Abbas et al., (2014). It is this ability to survive despite the application of pesticides that is of interest as it provides a mechanism to continue the transmission of diseases to both plants and animals. One of the most studied of the arthropods with a view of disease transmission are the mosquito species and research has shown that CYPs play a key role in the development of pesticide resistance (Corbel et al., 2007; Gong et al., 2005; Hemingway et al., 2004; Marcombe et al., 2009). Cytochrome P450s are split into clades depending on their function and this allows key CYPs to be more easily identified. Four main clades (mitochondrial clade, CYP2, CYP3 and CYP4 clade) exist and in terms of pesticide metabolism the clade that is of interest is the CYP3 clade, a clade which includes the important mammalian CYP3A4 and insect homologues of this enzyme, namely the CYP6 and CYP9 families e.g. CYP6Z1 (Chiu et al., 2008) and CYP6P3 (Muller et al., 2008).

Despite the importance of ticks, as they are second to mosquitoes as vectors of human disease and the most important vector of disease to animals (Hill and Wikel, 2005), with the ability to transmit a variety of pathogens and transmitting the widest array of disease causing organisms out of all the hematophagous arthropods (Bissinger and Roe 2010), little research has been carried out into the cytochrome P450 mediated metabolism and their subsequent associated resistance in this group. The vast majority of pesticide resistance research in these arthropods has focused on the role of GSTs (Kwon et al., 2010) and

carboxylesterases (Baxter and Barker 1998; Cossio-Bayugar et al., 2002), with the involvement of CYPs only being inferred by synergist studies (Li et al., 2004; Rodriguez-Vivas et al., 2013). Furthermore, despite the ever increasing problem of pesticide resistance little of this research has involved deciphering the mechanisms underlying the development of resistance phenotypes. The identification of CYPs, and indeed other drug metabolizing enzymes, in acari is hindered by the lack of available annotated genome data. Some data is available on public databases such as NCBI for *Ixodes scapularis* and the *I. scapularis* genome has been published (Van Zee et al., 2007) however for species such as *R. microplus*, the data available is even more sparse (Guerrero et al., 2006). It is therefore important to develop these resources such that a bioinformatics approach can be initiated to identify members of the key CYP families and where possible, identify putative homologues of the key insect CYPs that have been isolated and characterized to ascertain what they metabolize.

In order to be fully functional cytochrome P450s require an oxidoreductase (CPR in this case) and additionally in some cases cytochrome b_5. CPR is important in transferring electrons from NADPH through to the CYP allowing the incorporation of one molecule of oxygen, hence a monooxygenase reaction, to various substrates (Guengerich 2007). CPR is a highly conserved diflavin protein composed of two flavin cofactors, flavin mononucleotide (FMN) and flavin adenenine dinucleotide (FAD), that transfer the electrons one at a time to a CYP (Iyanagi et al., 2012; Lian et al., 2011). Due to the nature of CYP research among mammals and insects, the CPR gene has been isolated from an array of species and been characterized in many of these (Chen and Zhang 2014; Liu et al., 2014; Zhu et al., 2012) however, CPR has not been isolated or characterized from a tick species to date, despite the importance of this protein to the functioning of the monoxygenase complex and subsequent detoxification of xenobiotics. Differences have been identified

between human CPR and *An. gambiae* CPR which is potentially paving the way for a new target and method of control (Lian et al., 2011), but further emphasises the importance of characterising CPR isoforms from other arthropods.

Cytochrome b_5 is a small but highly conserved protein with a variety of roles in different reactions such as lipid and sterol biosynthesis (McLaughlin et al., 2010; Porter 2002; Zhang et al., 2005). The role cytochrome b_5 plays within the monooxygenase complex and CYP mediated reactions has been described as controversial for many years (Porter 2005) as cytochrome b_5 has been shown to have a stimulatory/modifying effect on CYP reactions, be it obligatory for some reactions and in other cases have an inhibitory effect (Im and Waskell 2011; Porter 2002; Schenkman and Jansson 2003). Despite this extensive amount of research into the role of cytochrome b_5 in CYP-mediated drug metabolism in mammals, much less equivalent research exists in arthropods considering the fact cytochrome b_5 may have a role in pesticide metabolism. Stevenson et al., (2012) found that by co-expressing numerous CYPs such as CYP9J2 from *Ae. aegypti* with cytochrome b_5 from *An. gambiae* the catalytic activity of the CYP was enhanced. Conversely, Chandor-Proust (2013) found that co-expression of *Ae. aegypti* cytochrome b_5 with *Ae. aegypti* CYP6Z8 did not cause a significant effect on the activity of the CYP (Chandor-Proust et al., 2013). This emphasises the need to co-express cytochrome b_5 when investigating CYP activity. Thus it is reasonable to assume that further investigation into the role of cytochrome b_5 in humans and some mosquito species will identify potential differences between species that similar to CPR, may additionally be exploited in the development of arthropod control measures.

In this paper we provide an update of our recent work towards filling the gap in the current knowledge of cytochrome P450s involved in pesticide metabolism in acari describing the isolation of the P450 monooxygenase complex from the Southern cattle tick, *R. microplus.*

Isolation of these individual components will allow detailed biochemical characterization and the ascertainment of their role in pesticide metabolism/resistance.

Materials & Methods

Bioinformatic analysis

115 putative cytochrome P450 sequences from *Rhipicephalus (Boophilus) microplus* provided by Dr Felix Guerrero and colleagues (Guerrero et al., 2006) were initially analyzed using the National Center for Biotechnology Information (NCBI) public database (http://www.ncbi.nlm.nih.gov/) to identify those having nucleotide sequence similarity to CYP3, CYP6 and/or CYP9. Of the 115 initial sequences 5 were identified as having the highest 'query coverage' and 'max ident' to known pesticide metabolizing CYPs from other species. Within these 115 were sequences were *R. microplus* homologues of cytochrome P450 oxidoreductase (CPR) and cytochrome b_5 ($cytb_5$) genes were also identified.

Tick samples

Tick larval samples from the species *R. microplus*, were provided from USDA, Texas (courtesy of Dr Felix Guerrero) preserved in RNA*later*-Ice® (Life Technologies, New York, USA), and stored at -80 °C to maintain RNA stability and integrity.

RNA Extraction and cDNA preparation

RNA was extracted from all 11 strains of *R. microplus* shown in Table 1 (the resistance profile of each strain is also included). The ticks were snap frozen using liquid nitrogen and RNA was extracted using TRIzol® reagent followed by a PureLink RNA Mini Kit (Life Technologies, New York, USA) according the manufacturer's instructions. RNA quantity

and purity was measured using a Nanodrop ND-100 (Nanodrop Technologies, Wilmington, USA) and integrity was checked using agarose gel electrophoresis.

cDNA was generated from the 11 strains of *R. microplus* ticks using PrimerDesign Reverse Transcription Premix (PrimerDesign, Southampton UK).

RACE Reactions

The Gonzales strain of *R. microplus* was used to generate RACE (Rapid Amplification of cDNA Ends) ready cDNA using a GeneRacer Kit (Life Technologies, New York, USA) and 1.44 µg of RNA was used to generate both 5' and 3' RACE ready cDNA. RACE PCRs were carried out using the RACE ready cDNA as follows:

5' RACE reactions contained, 300 µM dNTPs, 1 Unit VELOCITY DNA polymerase, 10 µl 5x amplification buffer, 900 nM GeneRacer 5' Primer (5' GGACACTGACATGGACTGAAGGAGTA 3') and 300 nM GSP Reverse primer (5' GGGCGAAGCCAGACGACACC 3'), 1 mM $MgCl_2$, 30µl sterile distilled water, 1 µl RACE ready cDNA template in a final reaction volume of 50 µl.

3' RACE reactions contained, 300 µM dNTPs, 1 Unit VELOCITY DNA polymerase, 10 µl 5x amplification buffer, 900 nM GeneRacer 3' Primer (5' GCTGTCAACGATACGCTACGTAACG 3') and 300 nM GSP Forward primer (10 µM) (5' TCCAGGTCCCCCACCAAGTTT 3'), 1 mM $MgCl_2$, 30µl sterile distilled water, 1 µl RACE ready cDNA template in a final reaction volume of 50 µl.

5' and 3' RACE fragments were generated using the following PCR cycling conditions: 1 cycle of 98 °C for 2 minutes, followed by 5 cycles of 98 °C for 30 seconds, 72 °C for 2 minutes, followed by 5 cycles of 98 °C for 30 seconds, 70 °C for 2 minutes, followed by 30 cycles of 98 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 2 minutes with a final

extension of 72 °C for 10 minutes. RACE products were double strand sequenced in order to facilitate the generation of gene-specific primers for amplification of the full length cDNA.

PCR amplification of full length CYP3006G8

PCR reactions were set up using VELOCITY DNA Polymerase (Bioline, London, UK) as follows; 0.5 µl of cDNA (per 12.5 µl reaction), 10 µl 5 x Hi-Fi Reaction Buffer, 400 µM dNTPs, 10 µM of each forward (5' CCGCACTCGCTCGAATCAT 3') and reverse (5' GCTGCTGTAGCAAACGATGTC 3') primers, 1 mM $MgCl_2$, 2 Units of polymerase enzyme in a total of 50 µl. Reactions were subjected to the following PCR cycling conditions. 1 cycle of 98 °C followed by 35 cycles of 98 °C for 30 seconds, 52 °C for 30 seconds and 72 °C for 1 minute with a final step of 72 °C for 10 minutes. Following PCR, products were analyzed on a 1 % agarose gel. The full length cDNA as cloned into pCR Blunt (Life Technologies, Paisley, UK) and the sequence confirmed via double strand sequencing using the vector specific M13 forward and reverse primers.

qPCR mRNA expression

cDNA was generated from the 11 strains of *R. microplus* ticks. This was carried out using Reverse Transcription Premix (PrimerDesign, Southampton, UK).

The full length CYP3006G8 was used to design a primer/probe set to carry out quantitative expression analysis: forward 5' TTTCCTTACTGCTTGCTTTCC 3', reverse, 5' CGTCAAAACACTTGTCTGCC 3', probe 5' FAM - TCATATCATTCGGTTCACGACATAACAAGACG – TAM. Additionally a primer/probe set was designed to ELF1α which was the housekeeping gene used to normalise the data (Nijhof et al., 2009): forward, 5' CTTCACCGACTTTCCTCCCCC 3', reverse 5' GCCTCCTTGGGTTTGACAGA 3', probe, 5' FAM - AAACGGTCGCCGTCGGTGTC – TAM

3'. Following optimization of primer/probe sets, for CYP3006G8 200 nM forward primer, 100 nM reverse primer and 300 nM probe were used in each reaction and for ELF1α 100 nM forward, 200 nM reverse and 300 nM probe was used. All reactions were set up as follows, 6.25 µl 2 x qPCR Master Mix for the iCycler (Primer Design, Southampton, UK), 2.5 µl RNase free water, primer/probe at the appropriate concentration indicated above and 5 ng/µl of cDNA. Reactions were then put through the following cycling conditions, 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds, 60 °C for one minute, data collection, using a BioRad CFX96 real-time PCR machine. The Ct values were converted into copy numbers (2^{-Ct}) and normalized against the corresponding values for the reference gene. Normalized values were subsequently compared to the reference strain (Gonzales) to determine the fold change in expression level of CYP3006G8 for each strain. Data was analyzed using the Students T-test to determine levels of significance.

Isolation of CPR and cytochrome b_5

Bioinformatic analysis allowed the 5' and 3' ends of the *R. microplus* CPR gene to be identified, allowing gene specific primers to be designed for use in PCR to amplify the full length cDNA. Reactions were set up as described above using the following primer set: forward, 5' CACGCGAGCCATGGAAGGGACG 3' and reverse, 5' GCAGCATCAACTCCAGACGTC 3'.

The full length cDNA of *R. microplus* cytochrome b_5 was identified via bioinformatics and primers were designed to amplify this gene. Reactions were set up as described above using the following primer set: forward, 5' CGTTCGCCAGTAGCTTTCCT 3' and reverse, 5' CCTGGTCTGTTTGCCCTCAT 3'. Both full length cDNAs were cloned into pCR Blunt and sequences confirmed via double strand sequencing using the vector specific M13 forward and reverse primers.

Peptide sequence analysis

Each of the isolated nucleotide sequences was analyzed using ExPASy translation software (http://web.expasy.org/translate/). This allowed the putative start and stop codons to be identified and determination of the open reading frame of each protein. It also allowed the identification of key residues and motifs to further confirm the isolation of each gene. The molecular weight of each protein was estimated using the ExPASy Bioinformatics Resource Portal and the compute pI/MW page (http://web.expasy.org/compute_pi/). Having used NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to investigate the homology of CYP3006G8 to other CYP proteins on the NCBI database and then using the same software to determine the homology of the putative CPR and cytochrome b_5 proteins to other similar proteins on the database, this allowed alignments to be carried out to show this homology. Alignments were carried out using Clustal omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Results

Isolation of CYP3006G8

Using known pesticide metabolizing CYPs as bait, bioinformatic analysis identified 5 potential homologues within the *R. microplus* transcriptome data set however during RACE-PCR only one set of primers designed to amplify sequence 60, successfully isolated both 5' and 3' products. Using these products gene specific primers were designed to amplify the full length cDNA of the encoded gene. The full length fragment was cloned, sequenced and submitted to Dr. David Nelson (University of Tennessee) for naming and has subsequently been recorded as CYP3006G8. The nucleotide and translated peptide sequence of

CYP3006G8 is shown in Figure 1, highlighting the key heme-binding domain essential for electron transfer and the membrane binding domain. CYP3006G8 has a calculated pI of 8.78 and a predicted molecular weight of 61.4 kDa.

Sequence homology analysis carried out on CYP3006G8 using NCBI Blastp confirmed homology to CYP6 and CYP9 sequences from several mosquito species (Table 2). Pairwise analysis found that CYP3006G8 shares maximum percent identities of 30.41 % to CYP6P3 (accession number AAL93295) from *Anopheles gambiae* and 30.14 % to CYP9J2 (accession number AAK17188) from *Aedes aegypti*. CYP6P3 has been shown to be involved in pyrethroid metabolism and resistance to pyrethroids (Muller *et al.,* 2008) and members of the CYP9J family have been shown to metabolize pyrethroids also (Stevenson *et al.,* 2012). Figure 2 shows an alignment of the protein sequences of these two mosquito CYPs along with CYP3006G8 and an additional NCBI top hit of CYP6l1 (accession number XP_003744550) from a species of mite (*Metaseiulus occidentalis*).

mRNA expression analysis

In order to gain further support for the hypothesis that CYP3006G8 is involved in pesticide metabolism, its mRNA expression levels in 11 different strains of *R. microplus* with varying resistance profiles was determined. Figure 3 shows the results of this analysis expressed as $\log_{10}$ fold change following normalization with ELF1α and using the Gonzales strain as the reference strain. The results of this analysis revealed that 8 of the 11 strains showed increased levels of expression of CYP3006G8 with the strains Cz, Co and Pq showing increased expression levels that were highly significant ($p = 0.0046$, $p = 0.0046$ and $p = 0.0056$, respectively). The Tx strain was also found to have an increase in mRNA expression level that was significant ($p = 0.027$). The strain Ca was found to have a significant decrease in mRNA expression ($p = 0.016$).

These results are interesting as the strains Cz, Co and Tx have all been shown to demonstrate resistance to pesticides, with Cz and Co displaying metabolic resistance to pyrethroids and Tx both target site and metabolic resistance to organophosphates. In addition the strain Pq has been shown to have target site resistance to pyrethroids but it is believed that metabolic resistance may also play a role in this resistance to pyrethroids. Conversely, the reduced mRNA expression level of the strain Ca also correlates with a role for CYP3006G8 in pyrethroid metabolism as this strain is susceptible to pyrethroids but resistant to organophosphates.

However this correlation is not entirely clear cut as Figure 3 shows the expression level of CYP3006G8 is highly variable across the 11 strains, in particular in the De strain which has been shown to demonstrate susceptibility to all classes of pesticides. As such, further biochemical analysis is required.

Isolation of CPR

Bioinformatic analysis of the transcriptome data set also identified the 5' and 3' ends of *R. microplus* CPR allowing the generation of gene specific primers and the isolation of a full length cDNA. The full length nucleotide and predicted peptide sequences of *R. microplus* CPR are shown in Figure 4. The encoded peptide has a predicted pI of 5.62 and a molecular weight of 77.8 kDa, a sequence alignment with CPR homologues from other species identified the conserved binding motifs for FMN, FAD and NADPH (Figure 5). The results of the pairwise analysis following the alignment are shown in Table 3 and clearly show the high degree of sequence homology amongst cytochrome P450 oxidoreductase from a variety of species but also shows that variation exists.

Isolation of Cytochrome b_5

A full length cDNA for *R. microplus* cytochrome b_5 was identified following bioinformatic analysis of the 115 transcriptomic sequences allowing this gene to be successfully amplified. The full length nucleotide and translated protein sequence are shown in Figure 6. The encoded protein has a predicted pI of 5.3 and a molecular weight of 15.1 kDa and displays a high degree of homology to other species as shown by the alignment in Figure 7, including the highly conserved histidine residues which are important for heme binding and residues important for creating the "*b_5 fold*". This high degree of homology is further illustrated by values obtained for pairwise analysis presented in Table 4. Figure 6 and Figure 7 illustrate the high number of acidic residues which generates the acidic nature of this protein.

Discussion

Pesticide resistance amongst the arthropods is known to be a major problem in both medicine and veterinary. There are a huge number of arthropods that act as vectors for disease and others that cause massive destruction of farm crops. Despite this there is still very little knowledge and understanding of how arthropods are able to develop resistance via metabolism of commonly used classes of pesticides such as pyrethroids and organophosphates. Cytochrome P450s through the monooxygenase complex have been shown to be a key mechanism of metabolic resistance to pesticides in numerous arthropods yet this area of research has not been well investigated in the Acari, especially the *Ixodidea* which includes the Southern cattle tick and the black-legged tick both of which transmit disease that affect humans and animals.

CYP3006G8 becomes only the third, cytochrome P450 to be isolated from the Southern cattle tick, *Rhipicephalus (Boophilus*) *microplus*, with the previous two being isolated over a decade ago. Crampton et al., (1999a) isolated a gene that was named Cyp4w1, however the authors used only sequence analysis and phylogenetics to investigate the gene isolated and did not carry out any biochemical characterization. Additionally, CYP4W1 was found not to be associated with pesticide resistance. He et al., (2002) isolated the gene Cyp319a1 from *R. microplus* and carried out semi-quantitative PCR and found no variation in its expression in six different strains with varying resistance profiles. Similar to CYP4W1, no heterologous expression or biochemical characterization has been carried out for CYP319A1.

Bioinformatic analysis suggests that CYP3006G8 displays some homology to CYP6 and CYP9 members although the level of homology to these key pesticide metabolizing CYPs is not greater than the 40% homology required to be placed in these families. Despite this, the results of the alignments as shown in Figure 2 and the mRNA expression analysis of CYP3006G8 in 11 *R. microplus* strains does provide support of a role of this novel CYP in pesticide metabolism.

The strains Cz, Co and Pq all of which have been profiled to display metabolic resistance to pyrethroids were found to have significant increases in the mRNA expression level of CYP3006G8. Additionally, the Tx strain which has been shown to have target site resistance and metabolic resistance to organophosphates showed an increase in expression of CYP3006G8 which despite being resistance to a different class still supports the hypothesis that this enzyme plays a role in metabolism/pesticide resistance. The other strain that had a significant difference in mRNA expression levels of CYP3006G8 was the Ca which displays target site and some metabolic resistance to organophosphates however is susceptible to all other classes including pyrethroids. This is of interest as this strain

displayed down-regulation of CYP3006G8 further supporting the hypothesis that CYP3006G8 is involved in pyrethroid pesticide metabolism. In view the data published for the previous two CYPs from this species, CYP4W1 (Crampton et al., 1999a) and CYP319A1 (He et al., 2002), CYP3006G8 is the first cytochrome P450 isolated from *R. microplus* that putatively metabolizes pyrethroids based on sequence homology and the correlation of mRNA expression with resistance profiling.

In addition to isolating a potential pyrethroid metabolizing CYP, the other components of the monooxygenase complex have also been isolated, the first of which is cytochrome P450 oxidoreductase (Figure 4) the sole electron transfer protein to CYPs. Isolation of this component is essential in determining not only the ability of CYP3006G8 to metabolize pyrethroids but also the activity of all future CYPs isolated from *R. microplus*. Recent work has been investigated the co-factor binding traits of CPR from different species, in particular between human and insects such as *Anopheles gambiae*. This is of interest as the comparison between the human and mosquito isoforms has identified differences in traits which may have far reaching implications (Lian et al., 2011). The results of this comparison found the concentration of the bound co-factors FMN and FAD per mol of enzyme were lower in *An. gambiae* than in the human CPR and that *An. gambiae* CPR was found to bind more weakly to 2',5'-ADP (an analogue to NADP) compared to human CPR. This latter finding is important as 2',5'-ADP can exert conformational changes which can affect the rate of electron transfer and thus affect the P450 catalysis. It is believed these subtle differences could be exploited to design new species specific pesticides. Isolating CPR for *R. microplus* will now allow similar studies to be carried out to identify if the same binding characteristics exits within all heamatophagous organisms.

The final component of the monooxygenase complex that was isolated was cytochrome b_5. Again, similar to CPR, this is the first reported isolation of this gene from *R. microplus*,

indeed from any acari species and completes the isolation of the monooxygenase complex from this species. Cytochrome b_5 is known to stimulate, inhibit or have no effect on catalysis by cytochrome P450s with much research carried out in mammalian CYPs (Finn et al., 2011; McLaughlin et al., 2010; Zhang et al., 2005), therefore it is important to be able to co-express cytb_5 to accurately determine the activity of CYPs with different substrates. The isolation of *R. microplus* cytb_5 will now allow co-expression with CYP3006G8 and any future CYPs that are isolated and expressed from *R. microplus* to determine the effect cytb_5 on catalysis and the role it may have in pesticide metabolism and the development of resistance. As no biochemical characterization has been carried out on CYPs from this species, there is no research showing what effect cytb_5 has and therefore this is an important aspect that requires future studies.

By employing the techniques used here and amplifying additional tick CYP genes attempts can continue to increase our knowledge and understanding of the role of cytochrome P450 genes in pesticide metabolism and ultimately the role they have in pesticide resistance. Identifying those CYPs involved provides the potential to investigate novel compounds (or even synergists) either natural such as plant derived products or synthetic that can be used to control tick populations in this case *R. microplus*.

Conclusions

Ultimately, this paper highlights the lack of research currently being undertaken in identifying cytochrome P450s in acari. This is despite the huge problem of pesticide resistance and the transmission of diseases to both humans and wildlife via ticks and mites acting as vectors. However using what data is available, we have been able to start identifying, isolating and characterising novel pesticide metabolizing CYPs and use the isolated monoxygenase components as an important tool in this area of research.

Characterising arthropod CYPs is an important process as it allows a greater understanding as to which pesticides may still have an effect due to susceptibility and which are unlikely to have any substantial effect due to the establishment of resistance in the field and additionally facilitates a more targeted based approach to the development of new pesticides.

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Table 1: The 11 strains of *R. microplus* along with the resistance profile of each strain used during expression analysis.

***R. microplus* Strain**	**Pesticide resistance or susceptibility**
Gonzalez (Gz)	Susceptible to all, very low level of metabolic resistance
Coatzoacoalcos (Cz)	Metabolic resistance to pyrethroids, target site pyrethroid resistance is absent, susceptible to other classes through low level of metabolic resistance to Organophosphates (OPs).
San Roman (SR)	Target site and metabolic resistance to OPs, susceptible to other classes.
Caporal (Ca)	Target site and metabolic resistance to OPs, susceptible to other classes.
San Alphonso (San Alfonso) (SA)	Metabolic resistance to amitraz probably. Target site resistance to pyrethroids.
Santa Luiza (SL)	Amitraz and pyrethroid resistance though mechanism unknown. Expect metabolic resistance is important
Corrales (Co)	Target site resistance to pyrethroids, some metabolic resistance to both pyrethroids and OPs also present.
Pesqueria (Pq)	Target site resistant to pyrethroids, probably some metabolic resistance to pyrethroids also present.
Deutsch (De)	Susceptible to OPs and pyrethroids.
San Felipe (SF)	Target site and metabolic resistance to pyrethroids. Probably low level of metabolic resistance to OPs.
Tuxpan (Tx)	Target site and metabolic resistance to OPs

Table 2: Pairwise analysis results. Results of pairwise analysis following alignment of CYP3006G8 with other arthropod CYPs shown in Figure 2

	Rm_CYP3006G8	M.occidentalis_CYP6l1	An.gambiae_CYP6P3	Ae.aegypti_CYP9J2
Rm_CYP3006G8	100.00	33.52	30.41	30.14
M.occidentalis_CYP6l1	33.52	100.00	25.10	23.96
An.gambiae_CYP6P3	30.41	25.10	100.00	31.09
Ae.aegypti_CYP9J2	30.14	23.96	31.09	100.00

Table 3: Pairwise analysis results. Results of pairwise analysis following alignment of *R. microplus* CPR with other CPR sequences

	H.sapiens	R.microplus	Ae.aegypti	D.melanogaster
H.sapiens	100.00	56.95	56.12	56.87
R.microplus	56.95	100.00	58.01	60.00
Ae.aegypti	56.12	58.01	100.00	78.85
D.melanogaster	56.87	60.00	78.85	100.00

Table 4: Pairwise analysis results. Results of pairwise analysis following alignment of *R. microplus* cytochrome b_5 with other cytochrome b_5 sequences

	H.sapiens	R.microplus	D.melanogaster	An.gambiae	Cu.quinquefasciatus
H.sapiens	100.00	51.16	46.15	44.44	46.46
R.microplus	51.16	100.00	50.00	48.82	53.12
D.melanogaster	46.15	50.00	100.00	60.94	67.44
An.gambiae	44.44	48.82	60.94	100.00	85.94
Cu.quinquefasciatus	46.46	53.12	67.44	85.94	100.00

Figure 1: Full Length sequence of CYP3006G8. Nucleotide and translated peptide sequence of the full length CYP3006G8 with the start and stop codons in bold/ underlined and characteristic heme binding domain (FxxGxxxCxG) bold/shaded. Membrane binding domain dashed underline.

Figure 2: Amino acid alignment of CYP3006G8 with other arthropod CYPs. CYP3006G8 aligned using Clustal omega with CYP6l1, CYP6P3 and CYP9J2 returned as top hits following Blastp searches. Heme-binding domain highlighted grey, ExxR motif boxed with solid line and PERF motif boxed with dashed line.

Figure 3: CYP3006G8 mRNA expression level as fold change compared to Gonzales. Fold change data transformed (Log_{10}). Strains Cz (P = 0.0046), Co (P = 0.0046), and Pq (P = 0.0056) showed highly significant difference (**) and strains Tx (P = 0.0027) and Ca (P = 0.016) showed significant difference (*). Strains labelled as shown in Tables 1.

Figure 4: Nucleotide and translated peptide sequence of *R. microplus* Cytochrome P450 Oxidoreductase. Data obtained following successful PCR amplification of CPR and subsequent sequencing. Start and stop codons in bold and underlined.

Figure 5: Protein alignment of *R. microplus* CPR with key regions highlighted. Alignment showing high degree of homology of CPR between different species. Membrane binding region dashed underlined. Key co-factor-binding regions are highlighted, FMN domains shaded grey, FAD domains boxed with a dashed line and NADP(H) domains boxed with a solid line.

Figure 6: Nucleotide and translated protein sequence of *R. microplus* cytochrome b_5. Data obtained following successful PCR amplification. Start and stop codons are in bold and underlined.

Figure 7: Peptide sequence alignment of *R. microplus* cytochrome b_5 and other arthropod and mammalian Cytb_5 sequences. An alignment showing the high degree of homology between species for the protein, cytochrome b_5. Conserved heme-coordinating histidine residues in the heme binding domain boxed. Residues highlighted grey indicates amino acids in the "b_5 fold". Membrane-binding domain dashed underlined.

Figure 1

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K L F L D E R H I K E K N L E L E S T F I F A
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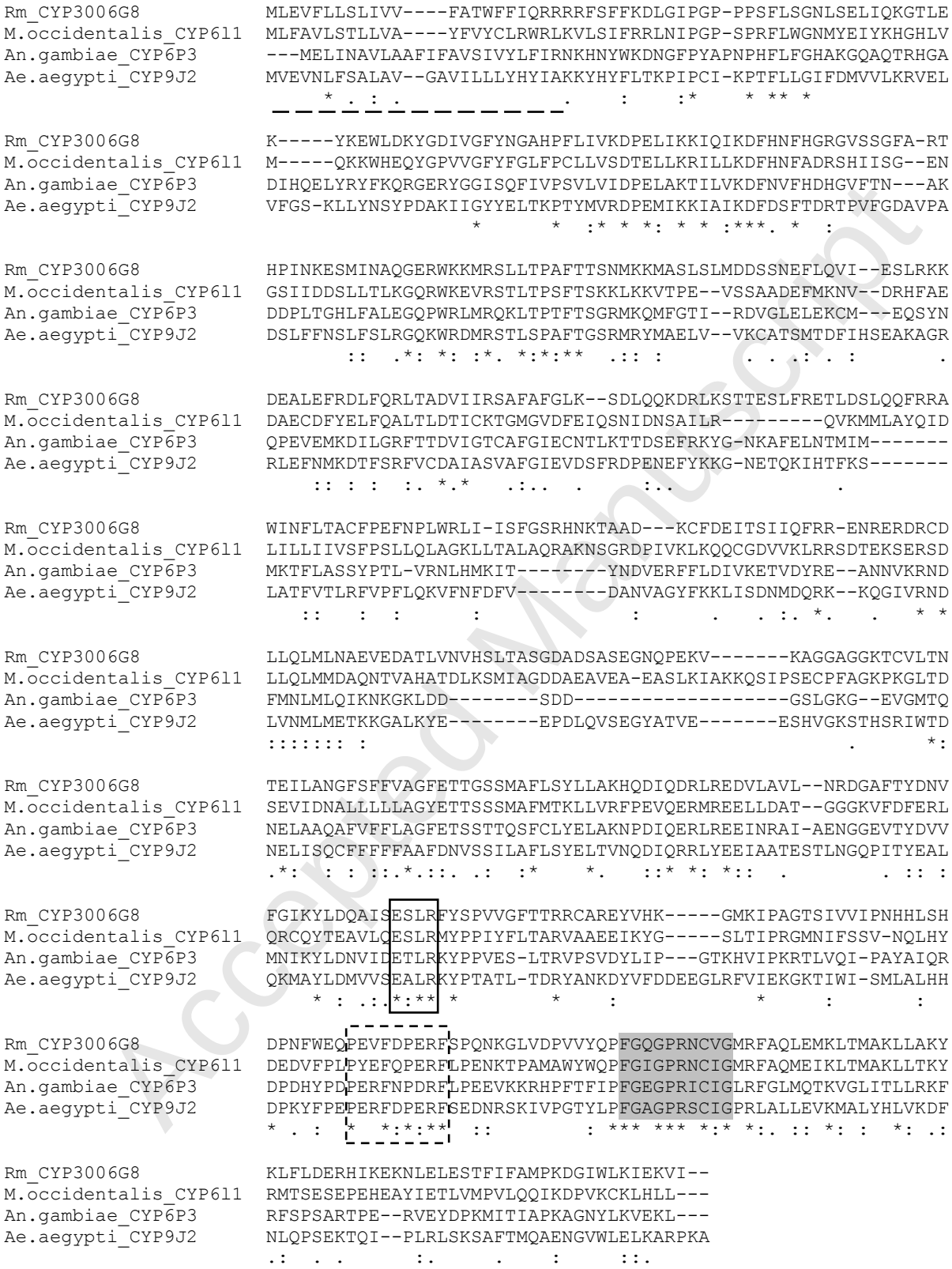

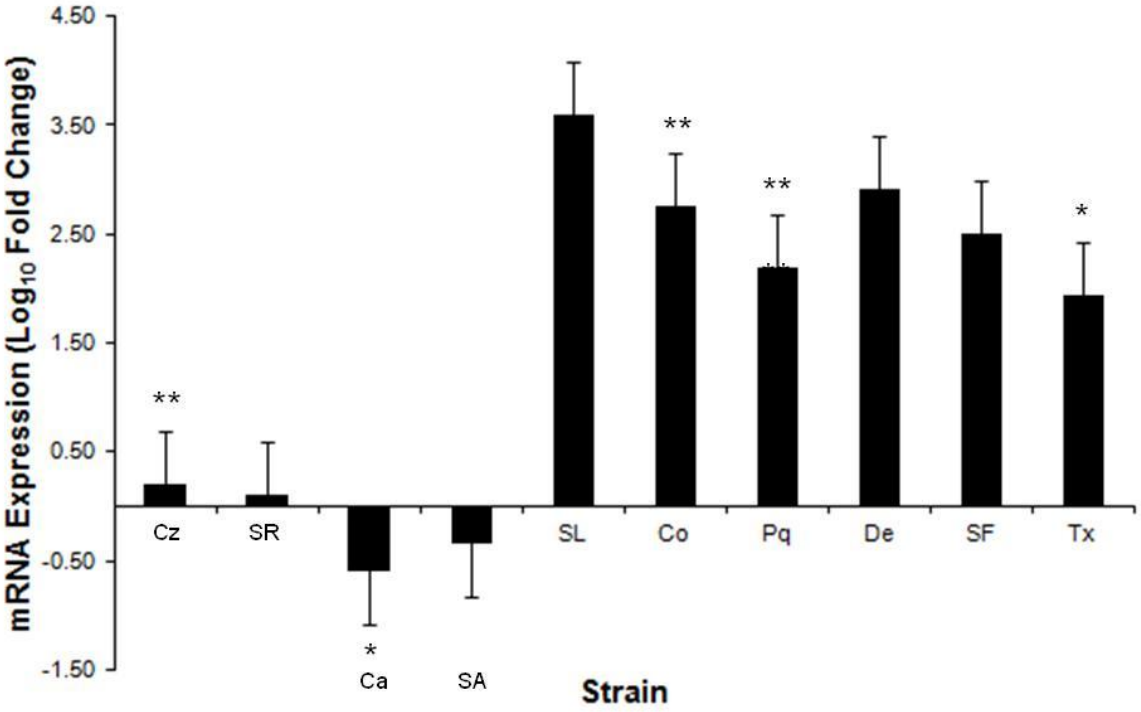

Figure 4

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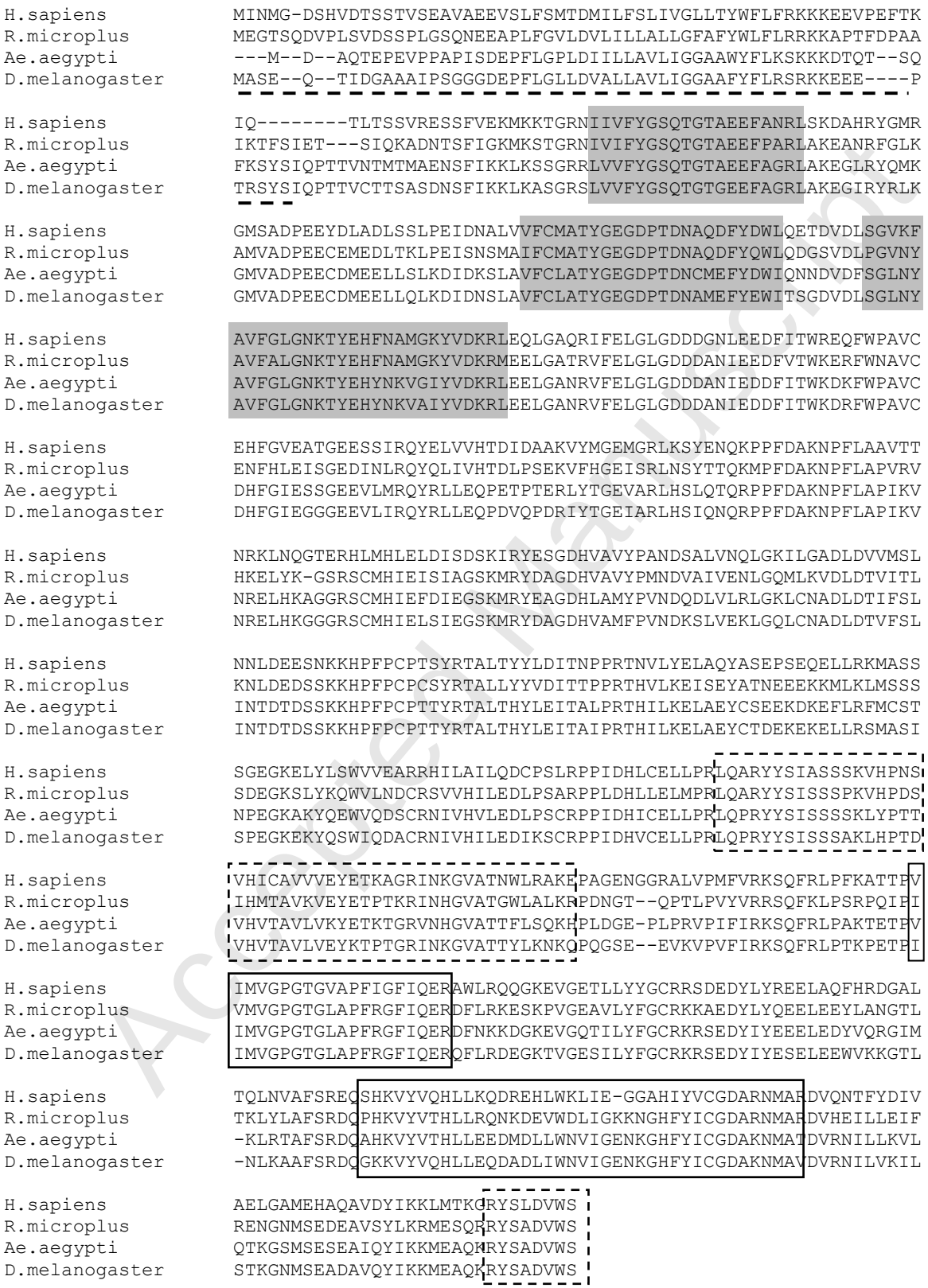

Figure 6

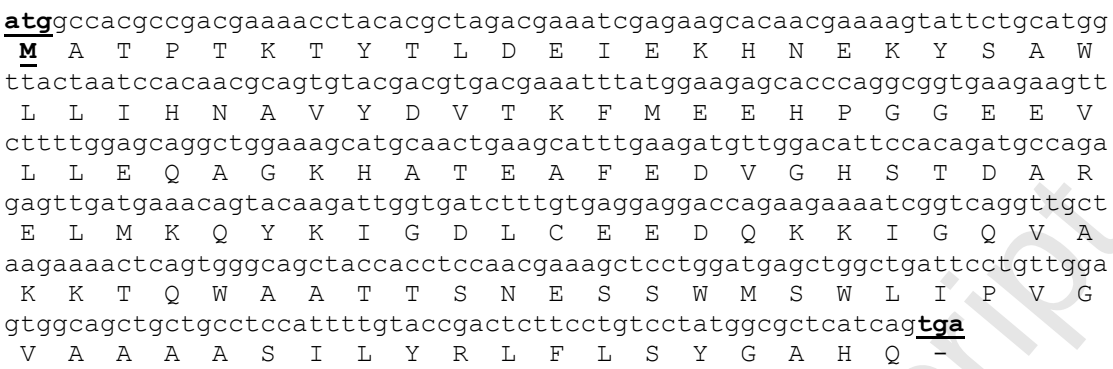

Figure 7