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Genetic and biochemical characterization of the cytochrome P450, CYP6P9, associated with pyrethroid resistance in the African malaria vector Anopheles funestus

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CHAPTER 2

Total Cytochrome P450 activity in *Anopheles funestus*

2.1 Introduction

Metabolism of some insecticides and natural toxic compounds is known to involve cytochrome P450-dependent monooxygenase enzymes. Over-expression of the P450 enzymes has been associated with pyrethroid resistance in *An. funestus* however, not much else is known about the enzymes themselves or the metabolism process of these detoxification enzymes (Amenya *et al*., 2008; Wondji *et al*., 2009; Cuamba *et al*., 2010).

Biochemical assays indirectly measure the levels of various enzyme activities, depending on the substrates and co-factors used and are carried out to determine the underlying metabolic mechanism. There are variants of biochemical assays, such as running in microtitre plates or using filter paper or nitrocellulose membrane, that have been successfully used on mosquitoes (*Anopheles, Culex* and *Aedes*), sand flies, cockroaches, houseflies and black flies as well as agricultural pests (WHO, 1998). These assays have successfully been used to measure the difference in specific enzyme activity between insecticide resistant and susceptible mosquitoes (Brooke *et al.*, 2001; Matambo, 2008). Significant increases in enzyme activity such as monooxygenases, esterases or Glutathione S-transferases (GSTs) provide insight into the specific metabolic resistance mechanism involved. This method was used by Brooke *et al.* (2001) to show that increased P450 activity was associated with permethrin resistance in *Anopheles funestus* from southern Mozambique. In addition to this, synergist studies using piperonyl butoxide (PBO) resulted in 100% mortality when resistant (FUMOZ-R) *An. funestus* mosquitoes were exposed to pyrethroids post PBO exposure, supporting the role of

monooxygenases in pyrethroid resistance (Brooke *et al.,* 2001). *In-situ* hybridization and quantitative trait locus (QTL) mapping by Wondji *et al.* (2007) also confirmed that the pyrethroid resistance locus was on chromosome arm 2R, mediated by one or a cluster of P450 enzymes specifically from the CYP6 gene family.

Several substrate models, such as 7-ethoxycoumarin, have been designed to monitor the activity of P450s. This is due to the fact that in the presence of P450 enzyme, 7-ethoxycoumarin is hydrolyzed to 7-hydroxycoumarin, which can be quantified by high-performance liquid chromatograph (HPLC) with fluorescence detection (Zting, 1981; Evans and Relling, 1992; Yamazaki *et al.,* 1999b). 7-ethoxycoumarin is metabolized by many P450 enzymes and has been used to monitor the level of P450 activity in mosquito microsomes (David *et al*., 2006; Matambo, 2008). However, this substrate has been known to have different rates of metabolism with various P450 genes and in various organisms, sometimes showing no metabolism at all (Yamazaki *et al*., 1999b). The ethoxycoumarin-O-deethlylase (ECOD) assay is a well used assay and is an indicator for oxidative metabolism (Frank *et al.*, 1997). Although biochemical assays can help to establish the resistance mechanism, they are not substitutes for standard WHO susceptibility tests. These enzymes might have increased activity for various other reasons outside of insecticide resistance (Scott *et al.,* 2003; Vontas *et al.,* 2005; Muller *et al*., 2007; Wood *et al.,* 2010).

Insecticide susceptibility analysis of adult mosquitoes is important for making informed decisions on biological control, insecticide use and alternatives. One of the methods available for the monitoring of susceptibility is the bottle bioassay method, introduced by Mc Allister and Brogdon (1999). The bottle bioassay provides the concentration of active ingredient of

pesticide per bottle for most organophosphate and pyrethroid insecticides and insects are then exposed to the insecticide by tarsal contact as they walk on it (Cochran, 1997; Brogdon and Mc Allister, 1998). The most widely used method is the standard WHO bioassay (WHO, 1998). Hargreaves *et al*. (2000) and Brooke *et al.* (2001) used the WHO insecticide susceptibility test and found that *An. funestus* was resistant to pyrethroids (Deltamethrin, 0.05%; Lambdacyhalothrin, 0.05%) as well as the carbamate propoxur (0.1%). It was concluded that resistance was due to mixed function oxidases (=monooxygenases) (Brooke *et al*., 2001). Mosquitoes under examination are generally compared with baseline (susceptible) populations, and tested with a diagnostic dose of active ingredient (Cochran, 1997; Brogdon and Mc Allister, 1998). The diagnostic dose is double the lowest concentration of insecticide that gives 100% mortality in susceptible mosquitoes over a one hour exposure (WHO, 1998; Peterson *et al*., 2004).

The objective of this study was two fold: firstly to conduct insecticide susceptibility testing on the various *An. funestus* strains that were to be used for further studies. The resistance profiles of these strains were determined using different types of pyrethroids, namely permethrin Type I (0.75%), deltamethrin Type II (0.05%) and lambda-cyhalothrin Type II (0.05%). Susceptibility testing was applicable to this study as it allowed for comparison to the response of known susceptible mosquitoes. The second part of the objective was to investigate oxidative metabolism via the cytochrome P450 assay, using 7-ethoxycoumarin as substrate and to compare levels of P450 activity across pyrethroid resistant (FUMOZ-R), susceptible (FANG) and unselected (FUMOZ) *An. funestus* strains.

2.2 Materials and methods

2.2.1 Laboratory strains of mosquitoes

Four laboratory strains of *An. funestus* were used for this investigation: 1) a pyrethroid susceptible strain (FANG), a colony originating from southern Angola; 2) a baseline strain (FUMOZ), originating from southern Mozambique; 3) a pyrethroid (permethrin) resistant strain (FUMOZ-R) selected from the baseline and routinely fed on guinea-pigs; and 4) FUMOZ-RH a sub-colony of FUMOZ-R, that is fed on human blood. These strains are routinely maintained in the Botha de Meillon insectary at the Vector Control Reference Unit (VCRU), National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa. All colonies are maintained in a standard insectary environment of 25 °C with 80 % relative humidity and 12 hour day/night, 45 minute dusk/dawn lighting cycle (Hunt *et al*., 2005).

2.2.2 Insecticide susceptibility tests

Insecticide susceptibility tests were performed prior to oxidative metabolism studies to confirm the resistance profiles of the four strains to different types of pyrethroids. The World Health Organization (WHO) standard protocols were used for insecticide susceptibility testing using filter papers impregnated with diagnostic doses of permethrin, Type I (0.75 %), deltamethrin, Type II (0.05 %) and lambda-cyhalothrin, Type II (0.05 %) (WHO, 1998). According to WHO (1998) criteria, <80% mortality 24hr post-exposure indicates resistance. Newly emerged FUMOZ-R, FUMOZ-RH and FUMOZ male and female adult mosquitoes were separated to prevent mating and placed into cages containing a 10% sugar solution. Ten replicates of three-day-old F_1 adults (n = 25) were each exposed to the aforementioned

insecticide-treated papers for 1 hour and the number of individual mosquitoes knocked down after 1 hour recorded. A 10% sucrose solution was made available to the mosquitoes and final mortalities 24 hours post-exposure were recorded for both males and females. The FANG strain, known to be susceptible to all insecticides, was used as a control to ensure the reliability of impregnated papers. If any papers showed less than 100% mortality, they were discarded from this experiment. Negative controls consisting of mosquitoes exposed to untreated papers were also used for both males and females under investigation to make sure that insectary conditions were not causing any unwanted deaths. The average mortality following 1 hour insecticide exposure and 24 hour recovery period for each sex and insecticidal treatment was calculated.

2.2.3 P450 assay

Protein extraction protocol: Preparation of microsomal and cytosolic protein fractions

Three-day-old adults (n = 500) were first decapitated because the eye pigment xanthommatin inhibits P450 activity (Danielson and Fogleman, 1994). The microsomal and cytosolic fractions were then prepared as follows, modified from the method described by Perry and Bucknor (1970): A crude protein preparation homogenizing buffer consisting of Na_2PO_4, pH 7.2 and the requisite protease inhibitors (1mM EDTA, 1mM DTT, 1mM PTU, 200mM sucrose, 1µg/ml aprotinin and 1µg/ml leupeptin) was prepared and kept on ice. The rotor was pre-cooled (-4ºC) for 1hr whilst the headless adult mosquitoes were homogenized with buffer. Test tubes were equilibrated and centrifuged at 10, 000 x g for 15 minutes and the supernatant collected. This was re-equilibrated and centrifuged at 100, 000 x g at 4ºC for 1hr. The resulting supernatant contained the cytosolic fraction, which was pipetted into falcon tubes and stored at -70ºC. The pellet contained the microsomal fraction and this was resuspended in

an appropriate amount of homogenizing buffer (dependent on the size of the pellet) and stored at -70ºC.

Determination of total protein content using BIO-Rad solution

The Bradford assay is a protein determination method that involves the binding of Coomassie brilliant Blue G-250 dye to proteins (Bradford, 1976). Bradford solution (BIO-Rad, cat no: 500-0006, South Africa) protein reagent was prepared as a 1:4 dilution in distilled water and added to a small volume of the crude homogenate. In addition, two controls were prepared in plastic cuvettes comprising distilled water and BIO-Rad solution. The absorbance for each sample was analyzed at 595nm (Shimadzu UV-160A Spectrophotometer) after 5 minutes incubation at room temperature. The optical density (OD) readings were expected to be between 0.1 and 1.0. Three replicates were performed for each sample tested and the average OD and protein concentration were determined using bovine serum albumen (BSA) as the reference standard (Appendix A1).

Oxidative metabolism studies:

i) 7-hydroxycoumarin standard curve

The substrate 7-ethoxycoumarin was used to assay P450 activity. 7-Ethoxycoumarin is a model monooxygenase substrate which is metabolized by a number of different forms of microsomal P450s (Frank *et al.*, 1997). In the presence of P450 enzyme, 7-ethoxycoumarin is hydrolyzed to 7-hydroxycoumarin. A standard curve was prepared using 7-hydroxycoumarin. A 0.1M sodium phosphate buffer pH 7.2 with 1mM EDTA; 1mM DTT; and 200mM Sucrose (Phosphate buffer) was added to various concentrations of 7-hydroxycoumarin, as performed by Matambo (2008). Samples were measured in a fluorospectrophotometer (Luminescence

Spectrometer LS50, Perkin Elmer) at an excitation wavelength of 390nm and an emission wavelength of 465nm and plotted on a graph against concentrations of 7-hydroxycoumarin.

ii) *Cytochrome P450 assay on An. funestus microsomal P450*

Total microsomal P450 protein, ranging from 0.4mg/ml – 2.7mg/ml was added to a microtitre plate (white walled). A mixture [as performed by Matambo (2008)] comprising of 0.1M Phosphate buffer, 20mM 7-ethoxycoumarin and 10mM NADPH was added to each well containing microsomal protein and incubated for 30 minutes at 30°C. A second mixture comprising of 30mM glutathione oxidized (GS-SG), 0.5 units glutathione reductase and water was added and allowed to incubate for 10 minutes at room temperature. The reaction was stopped with Tris-acetonitrile (50mM Tris pH 8; 1:1 acetonitrile). Samples were measured in a fluorospectrophotometer (Luminescence Spectrometer LS50, Perkin Elmer) at an emission of 465nm and excitation of 390nm. The amount of 7-hydroxycoumarin formed was calculated in comparison to a standard of 7-hydroxycoumarin in dimethyl sulfoxide (DMSO) and plotted on a graph [A465 against 7-hydroxycoumarin (μM)]. Controls included reactions with no NADPH and another with no enzyme (buffer only). Specific activity for all strains tested was expressed as μM/mg of P450 protein.

2.3 Results and discussion

2.3.1 Resistance characterization in Anopheles funestus

The insecticide susceptibility tests that are used to measure resistance showed that broad spectrum pyrethroid resistance was present in the selected *An. funestus* colonies (Figure 2.1). The results for FUMOZ-R were fairly uniform for all three pyrethroids tested. Percentage 24h mortality ranged between 3.6% (lambda-cyhalothrin females) to 12% (lambda-cyhalothrin males), indicating resistance in FUMOZ-R. The highest mortality rates in FUMOZ-RH were observed in the permethrin males (21.2%) and females (19.2%), and the least mortality seen in the lambda-cyhalothrin exposed males (4.8%) and females (2.0%). Both FUMOZ-R and FUMOZ-RH gave a zero percent 60min knockdown for all three pyrethroids tested. The unselected FUMOZ base colony displayed higher 24h mortality rates, ranging from 40.4% (lambda-cyhalothrin females) up to 95.6% (permethrin males). The 60min knockdown for FUMOZ was 94% (permethrin), 69% (deltamethrin) and 54% (lambda-cyhalothrin) whilst all FANG controls (males, n = 25; females, n = 25) showed 100% knockdown after 60min and 100% mortality after 24h. All negative controls (n = 25) on untreated papers survived (0% mortality).

These results indicated that the unselected baseline colony (FUMOZ) showed possible resistance to permethrin and was resistant to deltamethrin and lambda-cyhalothrin, despite being maintained for 10 years with no selection pressure. It's worthy to note that pyrethroid resistance in southern African *An. funestus* does not incur any loss of fitness under laboratory conditions, as shown by Okoye *et al.* (2007). The FUMOZ females showed more resistance than the males for all three insecticides tested, however there was no significant difference

(P<0.05), calculated using the two-sample Student's *t*-test assuming equal variances and 95% confidence intervals (CIs), P<0.05 (Statistix 7, Analytical software version 7.0). This was also observed for lambda-cyhalothrin exposed FUMOZ-R and FUMOZ-RH. FUMOZ-R and FUMOZ-RH permethrin and deltamethrin exposures however, did not follow this trend. Female mosquitoes showing more resistance than males has been observed as a common trend in these strains (Hunt *et al*., 2005; Wondji *et al.*, 2009) and might be linked to cuticle thickness (Wood *et al*., 2010). It has been postulated by Rewitz *et al*. (2006) and Baldwin *et al.* (2009) that increased resistance in females may be due to the hormone structure, which is dependent on P450s.

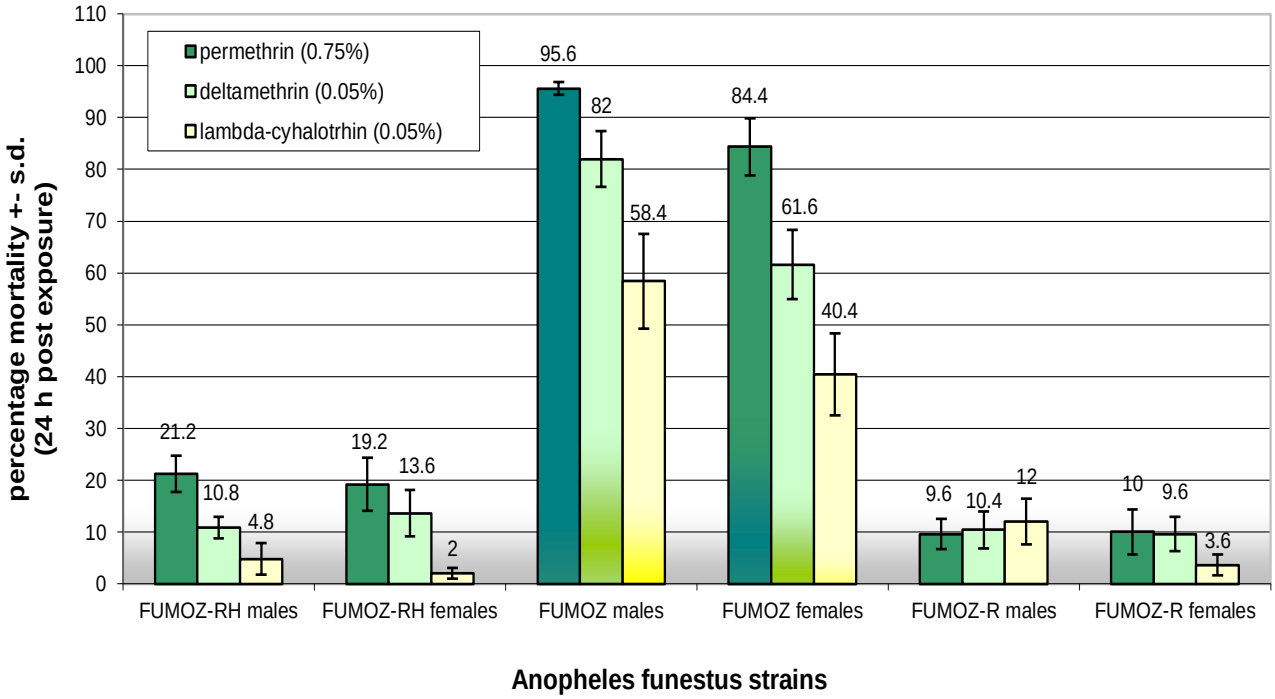


Figure 2.1: Insecticide susceptibility tests showing percentage mortalities and standard errors on 3-day-old F_1 *An. funestus* strains (FUMOZ-RH, FUMOZ and FUMOZ-R) following 60min pyrethroid chemical exposures (permethrin, deltamethrin and lambda-cyhalothrin) and a 24 h recovery period. Percentage mortality is calculated as a percentage of the total number of mosquitoes tested (n = 250) and are indicated above each bar. Mortality (98% – 100%) indicates susceptibility, 97% – 80% suggests possibility of resistance that needs verification, <80% indicates resistant. All FANG controls (males and females) showed 100% mortality (data not shown).

2.3.2 Cytochrome P450 activity assay

The cytochrome P450 assay using microtitre plates was used to investigate and compare levels of microsomal P450 activity between three mosquito strains. From the hydroxycoumarin standard curve (Figure 2.2), total microsomal P450 specific activity (μM/mg P450 protein) was calculated for males and females across FANG, FUMOZ and FUMOZ-R at the most productive time point of 10 minutes. Other time points (0 min, 2.5 min, 5 min) were investigated, however 10 min showed the most consistent data across three replicates, and also had the highest values as determined on the fluorospectrophotometer. To eliminate any variability in results, FUMOZ-RH was not used further in this study as its level of resistance was lower compared to FUMOZ-R and was more variable in mortality across the three pyrethroid chemicals tested. Two biological replicates (n = 500) were used and three technical repeats were performed. Due to sample size and time limitations it was feasible to do only two biological replicates.

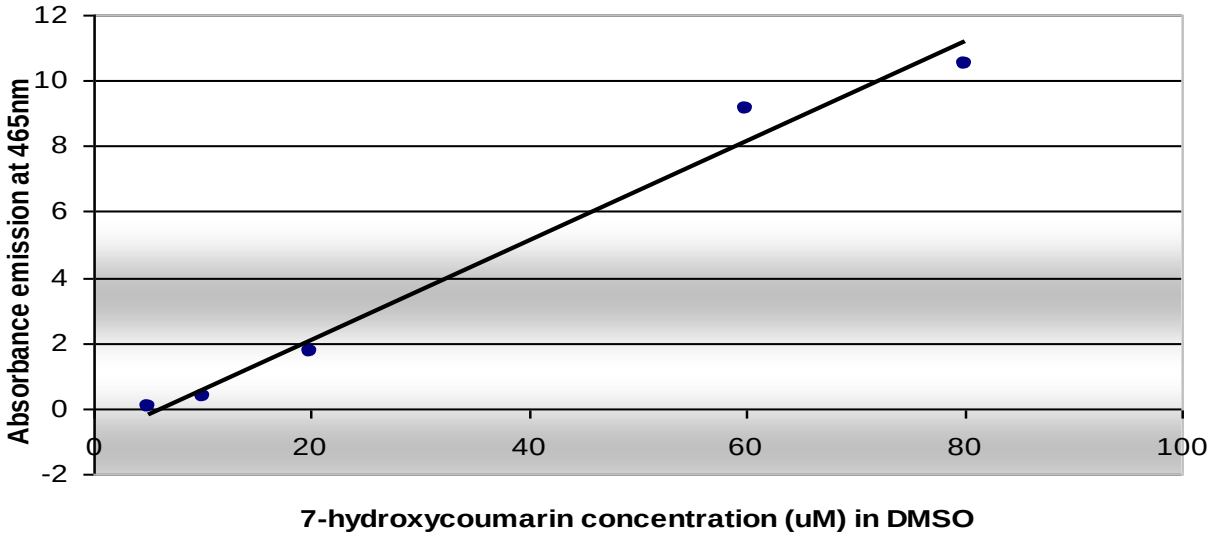


Figure 2.2: The 7-hydroxycoumarin standard curve was used to determine the concentration of 7-hydroxcoumarin formed from the hydrolysis of 7-ethoxycoumarin by microsomal P450 Proteins. The curve had an R^2 value of 0.9831 and the equation used was y = 0.1515x - 0.9491.

Results (Figure 2.3) indicated that FUMOZ-R females showed the highest level of P450 activity (1463.85 ± 138.46 µM/mg P450 protein) across the three strains. The FUMOZ-R males showed slightly lower activity (870.14 ± 113.14 µM/mg P450 protein) while FUMOZ males and females were similar in activity levels (246.87 ± 50.37 and 309.84 ± 34.62 µM/mg P450 protein respectively). The lowest level of activity was observed in FANG males and females (44.32 ± 2.26 and 47.30 ± 4.06 µM/mg P450 protein respectively) as to be expected. The FUMOZ-R females showed 4.7-fold greater P450 activity compared to the FUMOZ females and 30.9-fold greater P450 activity than the FANG females. The FUMOZ-R males showed 3.5-fold greater activity than the FUMOZ males and 19.6-fold greater activity compared to the FANG males.

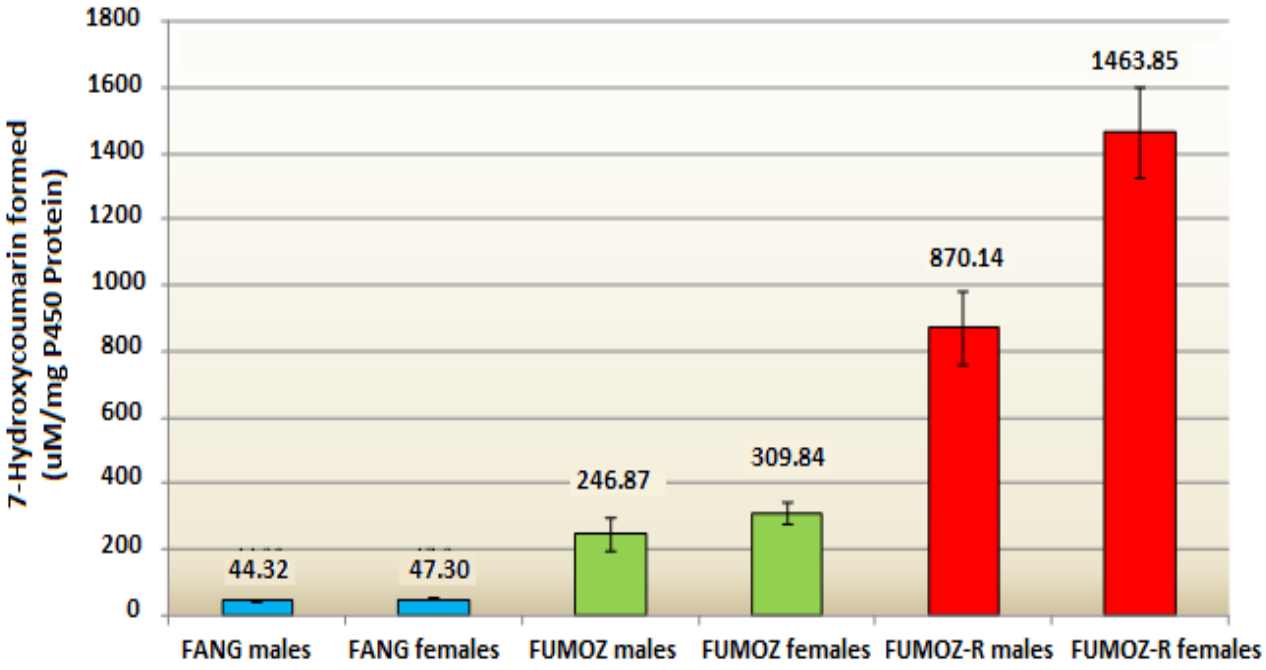


Figure 2.3: Total microsomal P450 assay showing specific P450 activity level of 7-hydroxycoumarin formed (µM/mg P450 protein) between males and females. FANG showed the lowest activity levels whilst FUMOZ-R, especially the females, showed the highest level of activity. When no enzyme was present and when NADPH was omitted from the reaction, very little if any 7-hydroxycoumarin was formed (data not shown).

These results correlate with the susceptibility data as increases in P450 activity (as seen in FUMOZ-R) have been related to increased resistance against pyrethroids (Brooke *et al*., 2001; Amenya *et al*., 2008) and very little P450 activity (as seen in FANG) related to their susceptibility to pyrethroids.

A combined effect of total P450 activity for males and females (Figure 2.4) was also investigated. The same trend seen in P450 activity confirmed the results seen above. FUMOZ-R, FUMOZ and FANG showed activity levels of 1166.99 ± 125.8, 278.36 ± 42.50 and 45.81 ± 3.16 μM/mg P450 protein respectively. FUMOZ-R showed 4.19-fold greater monooxygenase activity compared to FUMOZ and 25.5-fold greater monooxygenase activity than FANG. FUMOZ showed 6.07-fold greater monooxygenase activity compared to FANG. The negative controls (samples with no NADPH and one with buffer only excluding enzymes) showed no activity. Statistical analysis revealed that there was a significant difference ($P < 0.05$) between FUMOZ and FANG, FANG and FUMOZ-R, as well as FUMOZ-R and FUMOZ, calculated using the two-sample Student's *t*-test assuming equal variances and 95% confidence intervals (CIs), $P<0.05$ (Statistix 7, Analytical software version 7.0). The *t*-test results are given in Table 1. All results supported previously published data (Brooke *et al.*, 2001; Hunt *et al.*, 2005; Cuamba *et al.*, 2010; Morgan *et al.*, 2010) that P450s are the main metabolic resistance mechanism in *An. funestus*.

What is interesting to note is that natural populations of *An. funestus* from Tihuquine (Chokwe district) in Mozambique, only showed a 2.25-fold increase in the level of monooxygenase activity detected in this population compared to their *An.gambiae* control, the Kisumu strain ($P>0.05$) (Cuamba *et al.*, 2010). The fold increases observed in our laboratory-reared resistant

An. funestus strain (FUMOZ-R) is greatly elevated compared to this natural population, probably because of artificial selection. However, FUMOZ has never been selected to anything and is therefore more representative to a wild population than FUMOZ-R, and has 6.07-fold greater monooxygenase activity compared to the susceptible strain. This was an interesting finding and may be due to resistance alleles already present in this population. The use of the *An. gambiae* susceptible colony as a control with which to compare the fold increases observed in a different species by Cuamba *et al.* (2010) might also explain the low fold increase observed in their study.

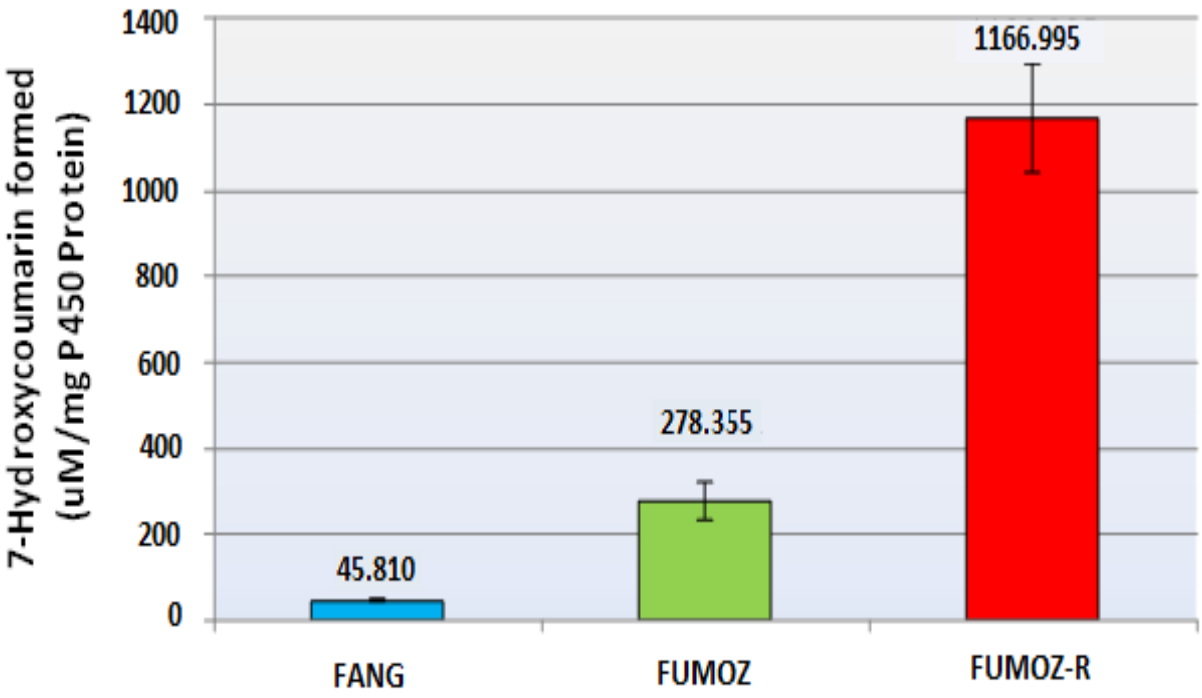


Figure 2.4: P450 assay on total microsomal P450 showing combined effect of 7-hydroxycoumarin formed between resistant, unselected and susceptible colonies. FUMOZ-R showed **4.19-fold** greater monooxygenase activity compared to FUMOZ and **25.5-fold** greater monooxygenase activity than FANG. FUMOZ showed **6.07-fold** greater monooxygenase activity compared to FANG.

Populations from Tihuquine showed no mortality (for both males and females) when exposed to 0.75% permethrin, 0.05% deltamethrin or 0.05% lambda-cyhalothrin for 1hr and 1hr 30min (Cuamba *et al*., 2010). There was also only a 30% mortality (deltamethrin) and 45% mortality (permethrin) when exposed for 3hr 30min, demonstrating extremely high resistance profiles, despite that in 2000 and 2002 this population of *An. funestus* from Chokwe was pyrethroid susceptible (Casimiro *et al.*, 2006; Casimiro *et al.*, 2007). Due to continued pyrethroid spraying in Maputo province, Mozambique (Sharp *et al*., 2007), selection pressure could have expanded into the Chokwe district and into the previously susceptible mosquito population. This might explain why these field populations of *An. funestus* in Mozambique are showing sharp increases in resistance.

Table 1: *t*-test analysis between P450 assay samples showing equal variances

Samples tested for t-test analysis (n = 500)	Equal variances (P*)	Test for equality of variances (P)
FUMOZ-R males versus females	0.000	0.426
FUMOZ males versus females	0.139	0.132
FANG males versus females	0.353	0.058
FANG males versus FUMOZ males	0.000	0.000
FANG males versus FUMOZ-R males	0.000	0.000
FUMOZ-R males versus FUMOZ males	0.000	0.054
FANG females versus FUMOZ-R females	0.000	0.000
FANG females versus FUMOZ females	0.000	0.000
FUMOZ females versus FUMOZ-R females	0.000	0.003
All FANG versus all FUMOZ	0.000	0.000
All FANG versus all FUMOZ-R	0.000	0.000
All FUMOZ-R versus all FUMOZ	0.000	0.000

*** where P = probability, calculated using the two-sample Student's *t*-test assuming equal variances and 95% confidence intervals (CIs), P<0.05.**

2.4 Conclusion

Susceptibility testing against three different pyrethroid chemicals, for both males and females and across susceptible, unselected and resistant strains of *An. funestus*, showed that the females of all three strains were generally more resistant than the males for all three insecticides tested, except for permethrin-treated females (FUMOZ-R) and deltamethrin-treated females (FUMOZ-RH). The role of P450s in resistance was analyzed using the cytochrome P450 assay to investigate and compare levels of P450 activity in microsomes isolated from headless adult mosquitoes, across the three strains. Microsomes were isolated from 3-day-old adults, fed only on a 10% sucrose solution. The cytochrome P450 assay using 7-ethoxycoumarin as substrate demonstrated that monooxygenase activity was occurring and that enzyme activity in *An. funestus* microsomes is NADPH-dependent. High levels of enzyme activity were observed in the resistant strain (especially in the FUMOZ-R females), moderate activity in the unselected strain and very low P450 activity levels in the susceptible FANG strain. FUMOZ-R showed 4.19-fold greater monooxygenase activity compared to FUMOZ and 25.5-fold greater monooxygenase activity than FANG. FUMOZ showed 6.07-fold greater monooxygenase activity compared to FANG. Increased P450 activity is associated with increased resistance in *An. funestus* mosquitoes. Laboratory-reared *An. funestus* may not reflect the true P450 activity present in natural field populations of *An. funestus*, but it is a model to study this mechanism in more detail. Identifying candidate genes involved in insecticide resistance and assessing their gene expression and protein expression profiles may help elucidate the main mechanism of pyrethroid resistance further. Characterizing the specific P450s might aid in the identification of specific markers for detecting pyrethroids rapidly in the field.