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# Age-related pyrethroid resistance is not a function of P450 gene expression in the major African malaria vector, *Anopheles funestus* (Diptera: Culicidae)

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**ABSTRACT.** *Anopheles funestus* is a major vector of malaria in most of the African region. Resistance to pyrethroid and carbamate insecticides has been recorded in populations of this species in South Africa and Mozambique. The P450 gene, *CYP6P9*, has been shown to be highly transcribed in a permethrin (pyrethroid)-resistant laboratory strain, FUMOZ-R, originating from southern Mozambique. We examined the relationship between pyrethroid resistance and gene transcription levels of two closely related genes, *CYP6P9* and *CYP6P13*, in FUMOZ-R. Levels of resistance to 0.75% permethrin were determined based on standard WHO insecticide susceptibility assays using females and males of different ages, ranging from 3 to 30 days old. The transcription

 

levels of the two genes were quantified using qPCR for each age cohort. In the WHO insecticide susceptibility assays, survival of both males and females significantly decreased as age increased. Quantitative analysis of the two genes *CYP6P9* and *CYP6P13* showed the highest levels of expression at 10 days of age. There was no correlation between expression of these two genes and pyrethroid survival by age. We conclude that the resistance of this mosquito strain to permethrin is not directly related to age-mediated differences in *CYP6P9* and *CYP6P13* expression.

**Key words:** *Anopheles funestus*; Age; Pyrethroid resistance; P450 expression

## INTRODUCTION

Malaria affects an estimated 247 million people worldwide resulting in approximately one million deaths each year (WHO, 2008). Ninety percent of deaths due to malaria occur in Africa. Malaria is transmitted by *Anopheles* mosquitoes including the major African vector *Anopheles funestus*, which transmits malaria perennially and has also been associated with malaria epidemics (Gillies and De Meillon, 1968; Fontenille et al., 1990; Hargreaves et al., 2000).

Effective malaria vector control is potentially hampered by the development of resistance to insecticides. Therefore, an understanding of resistance mechanisms in vector populations enables the development of resistance management strategies. The most common modes of insecticide resistance are reduced target-site sensitivity and enzyme mediated detoxification. Target-site insensitivity mutations result in reduced affinity of the target receptor to insecticide (Brogdon and McAllister, 1998). Alterations of the sodium ion channel gene (*kdr*), the γ amino-butyric acid (GABA) receptor and acetylcholinesterase (AChE) reduce the binding affinities of pyrethroids and DDT, dieldrin and fipronil, and carbamates and organophosphates, respectively (Hemingway and Ranson, 2000).

The first account of insecticide resistance in *A. funestus* in southern Africa and its impact on malaria transmission were reported in 2000 (Hargreaves et al., 2000). Pyrethroid resistance in *A. funestus* from South Africa and southern Mozambique was subsequently shown to be mediated by a monooxygenase (P450) detoxification mechanism and no target site insensitivity was identified (Brooke et al., 2001; Wondji et al., 2007; Okoye, 2008). Recently, the molecular characterization of monooxygenase-based pyrethroid resistance in *A. funestus* was initiated (Amenya et al., 2005, 2008; Wondji et al., 2007, 2009; Matambo et al., 2010).

Amenya et al. (2005) isolated the first 31 partial monooxygenase P450 sequences and subsequently reported that one of these, *CYP6P9*, was over-expressed in the egg and adult stages of a pyrethroid-resistant laboratory strain (FUMOZ-R) originating from southern Mozambique (Amenya et al., 2008). Wondji et al. (2007) identified a quantitative trait locus (QTL) associated with pyrethroid resistance in the same *A. funestus* strain and confirmed the increase in *CYP6P9* transcription. These QTL markers contained a cluster of P450 genes including *CYP6P9*. Subsequently, Matambo et al. (2010) and Wondji et al. (2009) reported on a duplicate gene and called these genes *CYP6P13* and *CYP6P9b*, respectively. Amino acid similarities between *CYP6P13* and *CYP6P9b* confirm that these are the same genes (Wondji

et al., 2009; Matambo et al., 2010). As *CYP6P13* was officially named by the P450 Nomenclature Committee (Dr. Nelson, http://drnelson.uthsc.edu/CytochromeP450.html) during 2009 (Matambo, 2008) our paper will retain the name *CYP6P13* for this gene.

Gene transcription studies by Amenya et al. (2008) and Wondji et al. (2009) did not differentiate between these two closely related genes as the primers used for both these studies were designed in a region sharing 100% sequence identity between them. Recent studies by Morgan et al. (2010) confirmed the increased mRNA expression of both these genes in *A. funestus* from Uganda while Cuamba et al. (2010) reported on the overexpression of one of the duplicate genes (*CYP6P9a*, called *CYP6P13* in our article). Furthermore, a recent microarray study by Christian et al. (2011) showed an increase in gene transcription for these genes. Only one other P450 gene was found to be upregulated (*CYP6M7*) but this gene was not validated using quantitative real-time PCR (qPCR) (Christian et al., 2011).

As mosquitoes are infective and able to transmit malaria only after age 10-14 days, it is important to characterize insecticide resistance in older mosquitoes. The aim of this project was to quantify the levels of pyrethroid resistance in a highly resistant strain of *A. funestus* males and females at different ages (3, 5, 10, 14, 20, and 30 days) as well as to quantify the transcription levels of the two duplicated genes, *CYP6P9* and *CYP6P13*, in the same age cohorts in order to ascertain whether there is a direct relationship between gene transcription and pyrethroid resistance phenotype by age.

## MATERIAL AND METHODS

### Mosquitoes

The FUMOZ-R *A. funestus* laboratory colony originates from southern Mozambique and was selected for high levels of resistance to permethrin (pyrethroid). Details of colony rearing, permethrin resistance selection and insectary conditions can be found in Hunt et al. (2005). Female and male adults were separated on emergence and were maintained on a 10% sugar solution until they reached the ages of 3, 5, 10, 14, 20, or 30 days. A separate cohort of female adults were allowed to mate after emergence and were offered blood meals when they were 6, 8 and 10 days of age. On day 14, they were used either for insecticide susceptibility testing or for RNA extractions.

### Evaluation of the influence of *A. funestus* age on their pyrethroid resistance

The insecticide susceptibility assays described in this study were carried out according to WHO specifications using WHO insecticide-treated papers (WHO, 1998). The insecticidal action of all test papers was first confirmed using samples of an *A. funestus* susceptible strain (FANG), which showed 100% mortality following exposure to all papers.

Approximately 30 FUMOZ-R adults per replicate were assayed against 0.75% permethrin-treated papers through 10 replicates for each age and sex cohort (total of 300 mosquitoes per age group). For each replicate an untreated control was included. Final mortalities were recorded 24 h post-exposure and subsequent analysis was conducted on these data (WHO, 1998). As sample sizes were large throughout ($N \leq 290$), data were not transformed prior to analysis.

 

## Quantification of *CYP6P9* and *CYP6P13* transcripts for each age cohort

### *Total RNA extraction and cDNA synthesis*

Total RNA was extracted from 15 adult *A. funestus* FUMOZ-R mosquitoes to form one biological sample. Three biological extractions (N = 15 mosquitoes per biological sample) were performed per age and sex. The extractions were carried out using the Tri-reagent (Sigma, T9424, Munich, Germany). The RNA samples were DNase treated (Qiagen, 79254, West Sussex, UK), and were quantified using a Nanodrop® Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Total RNA (up to 2 µg) was converted to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Cat. No. 4387406, USA). The quality and quantity of cDNA was measured using the Nanodrop® Spectrophotometer (Nanodrop Technologies) and the cDNA samples were stored at -70°C until further use.

### Primer design

Two sets of primers were used in this study, one for *CYP6P9* (gene-specific primers) and the other for *CYP6P13* (gene-specific primers). The ribosomal protein 7 or rps 7 (GenBank accession No. EF450776) primers were used as a reference gene to normalized data. This gene was tested prior to analysis to confirm that transcription of this gene does not change with age.

The *CYP6P9* (GenBank accession No. EU450763) and *CYP6P13* (GenBank accession No. EF152577) primers were designed in the regions containing nucleotide base pair differences between the two genes and these primers are therefore specific for these genes. These primers were designed using the full-length *CYP6P9* and *CYP6P13* gene sequences (Matambo et al., 2010) (Table 1).

### Real-time quantitative PCR

qPCRs were carried out using the Bio-Rad CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). A total volume of 25 µL containing 12.5 µL IQ™ SYBR supermix (Bio-Rad, 1708882), 4 µL primer (2.5 µM), 1 µL cDNA (50 ng) was used per reaction. A standard curve reaction containing a 2-fold serial dilution of cDNA for each of the genes of interest and the house keeping gene was generated in each reaction. Serial dilutions of cDNA for the standard curve were prepared (Paton et al., 2000; Bio-Rad, 2006). This was correlated to the initial cDNA template used in the reaction and then converted to transcript copy number (tCNR) (Bio-Rad, 2006).

The cycling conditions for each primer set differed from each other. These conditions are presented in Table 1. Triplicates of each biological sample were carried out in each plate and the qPCR for the three different biological extractions was performed on different days. The reference gene, rps 7, did not vary between the different ages when amplified. The identity of all amplified products was confirmed by sequence analysis by sequencing in both directions. The sequenced genes were blasted against the *CYP6P9* and *CYP6P13* gene sequences to ensure that the correct product was amplified during qPCR.

**Table 1.** Primers used in quantitative real-time PCR.

| Gene | Primer sequence | Fragment length (bp) | Ta (°C) | Tm (°C) |
|---|---|---|---|---|
| CYP6P9 | Fwd 5' AGA TGT GAT TGG CAC CTG T 3' | 232 | 55.00 | 82.00 |
| (GAN: EU450763) | Rev 5' TCG ATA TTC CAC CGT TTC CT 3' | | | |
| CYP6P13 | Fwd 5' CTG GAT CTC CTA ATT ATG ATG AAG TTT TTC 3' | 132 | 59.10 | 81.00 |
| (GAN: EF152577) | Rev 5' GTT CAC CGT CTC GCG GAC T 3' | | | |
| rsp 7 | Fwd 5' TTA CTG CTG TGT ACG ATG CC 3' | 135 | ** | 85.50 |
| (GAN: EF450776) | Rev 5' GAT GGT GGT CTG CTG GTT C 3' | | | |

GAN = Gene accession number; Ta = annealing temperature; Tm = melting temperature. **Annealing temperature for the rsp 7 gene was 55°C when testing for CYP6P9 and 59.10°C for CYP6P13 analysis.

The data were analyzed using the absolute quantification method, using the formula:

$$N_n = 10 (n - b) / m,$$

where $n$ = Ct, $b$ = y intercept, $m$ = slope of line (Bio-Rad, 2006). The values were converted into tCNR and the ratio of each gene to the rps 7 transcription was determined (Bio-Rad, 2006). Standard errors (SE) were calculated for each replicate.

The Pearson's correlation statistical method was implemented to deduce if there was any association between the bioassay and the qPCR data.

## RESULTS

### WHO insecticide bioassays

Percentage survival of FUMOZ-R-resistant adults 24 h post-exposure to 0.75% permethrin was calculated by sex for each age cohort (10 repeats of ±30 mosquitoes per tube) (Figure 1). There was a significant trend in mortality with age in females (linear regression: P = 0.04; $R^2$ = 0.59) and males (linear regression: P = 0.02; $R^2$ = 0.60). In both cases, survival tended to decrease with age. Three-day-old females showed a mean survival of 78.1% (SE = 8.48), which decreased steadily through ages 5 (71.71%; SE = 8.64), 10 (68%; SE = 7.96) and 14 days (37.70%; SE = 5.77), showing that female susceptibility to permethrin increased with age (Figure 1A). The lowest survival was recorded in the blood-fed female cohort at 14 days (32.70%; SE = 7.26), although a two sample *t*-test revealed that there was no significant difference between blood-fed and -unfed females at this age (P = 0.64). Survival increased in the female cohorts at 20 and 30 days old. There was no significant difference between unfed 14- and 20-day-old females (P = 0.08).

A similar trend was observed in males (Figure 1B). The males' resistance to permethrin was high at ages three (71.81%; SE = 3.97) and five days (77.86%; SE = 7.68). At 10 days the percentage survival decreased to 48.83% (SE = 8.85) and then steadily decreased to a low of 26.84% (SE = 5.13) at age 14. The lowest survival in the males was recorded at age 20 (17.40%; SE = 1.89). There was no significant difference between 14- and 20-day-old males (P = 0.09). The percentage survival increased to 26.40% (SE = 4.28) at 30 days. There was a significant difference in percentage survival between 3- and 14-day-old males based on a two sample *t*-test (P = 0.00).

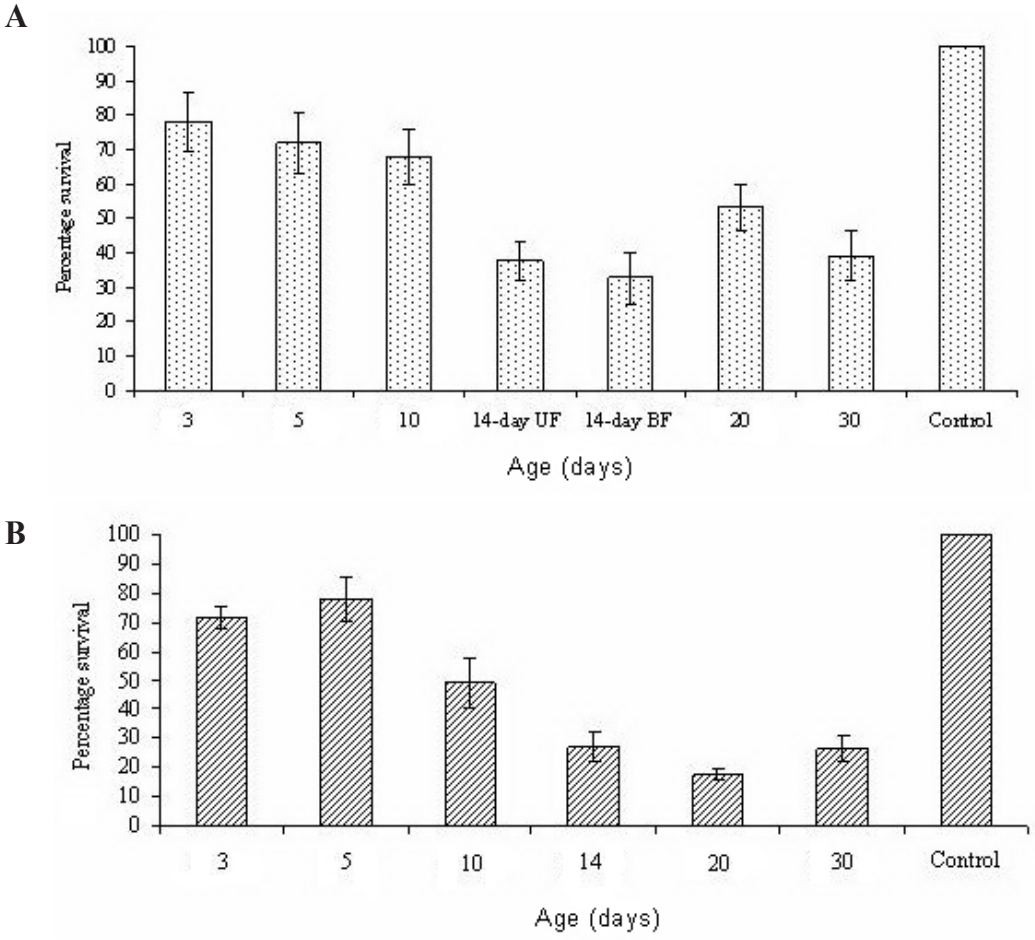


**Figure 1.** Percentage survival by age of pyrethroid resistant *Anopheles funestus* (FUMOZ-R) in females (**A**) and males (**B**) 24 h post-exposure to 0.75% permethrin. Standard errors (SE) are indicated on the bars. Control = exposure of mosquitoes to untreated papers. UF and BF = blood-unfed and blood-fed females, respectively.

## Gene transcription analysis of *CYP6P9*

In 3-day-old females, the tCNR was 1.88 (SE = 0.40). In both pyrethroid resistant females and males, the tCNR was high, 9.3 (SE = 3.08) at day 10 for females and 20.1 (SE = 1.08) for males at the same age, but decreased by day 14 to 3.63 (SE = 1.08) for females and 12.1 (SE = 3.46) for males (Figure 2). However, the tCNR then decreased to 3.63 (SE = 1.08) for unfed females and 1.74 (SE = 0.45) for blood-fed females at day 14, although there was no significant difference in tCNR between blood-fed and -unfed cohorts (two sample *t*-test: P = 0.18). The tCNR increased again at day 20 to 3.54 (SE = 1.8) and then decreased to 0.52 (SE = 0.20) at day 30.

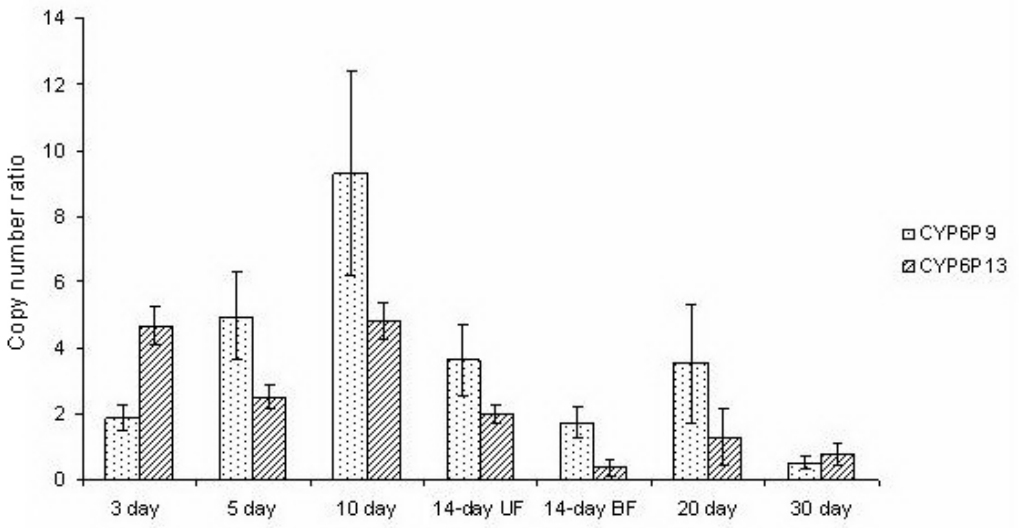


**Figure 2.** *CYP6P9* and *CYP6P13* transcription analysis by age, expressed as copy number ratio, in pyrethroid-resistant *Anopheles funestus* FUMOZ-R females. Standard error bars are indicated.

Male tCNR was 9.15 (SE = 3.87) at three days and increased to 23.9 (SE = 7.63) at five days followed by a steady decrease of 20.1 (SE = 1.08) at 10 days, 12.1 (SE = 3.46) at 14 days, 5.77 (SE = 1.83) at 20 days, and 6.56 (SE = 2.02) at 30 days (Figure 3). Overall, there was no significant difference in the absolute transcription level of *CYP6P9* in association with age (ANOVA: females, P = 0.17; males, P = 0.07). There was no significant correlation between survival (as quantified by insecticide bioassay) and gene transcription levels for *CYP6P9* (linear regression: females, P = 0.46, $R^2$ = 0.18; males, P = 0.16, $R^2$ = 0.37).

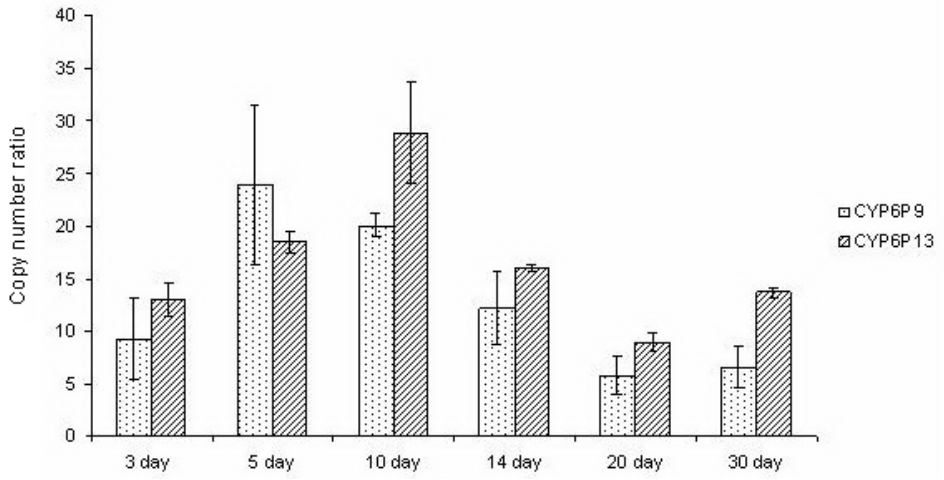


**Figure 3.** *CYP6P9* and *CYP6P13* transcription analysis by age, expressed as copy number ratio, in pyrethroid-resistant *Anopheles funestus* FUMOZ-R males. Standard error bars are indicated.

## Gene transcription analysis of *CYP6P13*

Gene transcription levels of *CYP6P13*, using the absolute quantification method, revealed that the tCNR changed only slightly from 4.69 (SE = 0.60) in 3-day-old females to 4.82 (SE = 0.56) (Figure 2) in 10-day-old females, while males of the same age had a tCNR of 28.84 (SE = 4.88) (Figure 3). However, the tCNR almost halved between 3- and 14-day-old unfed females although the difference is not statistically significant (two sample *t*-test: P = 0.15). The tCNR of the gene had decreased from 2 (SE = 0.27) at 14 days when the females were not given a blood meal to a significant 0.38 (SE = 0.24) when the females had access to a blood meal (P = 0.01). The tCNR increased to 0.79 (SE = 0.32) by day 30 in females and had increased from 8.92 (SE = 0.91) to 13.67 (SE = 0.53) in males by age 20. Overall, there was no significant difference in the absolute transcription levels of *CYP6P13* in association with age (ANOVA: females, P = 0.39; males, P = 0.6). There was no significant correlation between survival and gene transcription levels for *CYP6P13* (linear regression: females, P = 0.06, $R^2$ = 0.62; males, P = 0.52, $R^2$ = 0.11).

## DISCUSSION

Variation in the expression of insecticide resistance with age is shown here for *A. funestus* and has also been described in other anophelines. Hunt et al. (2005) describe fluctuations in the expression of pyrethroid resistance with age in unselected and selected generations of FUMOZ-R. The effect of age may lead to a weakening of resistance mechanisms as a consequence of a general progressive decrease in physiological capacity (Hodjati and Curtis, 1999). This could in turn result in the mosquitoes having reduced ability to respond or adapt to environmental changes.

Successive blood feeding prior to reaching 14 days old did not affect female susceptibility to pyrethroid intoxication as assessed four days after the last blood meal. However, Spillings et al. (2008) showed that blood-feeding significantly enhanced resistance to pyrethroid intoxication in the FUMOZ-R strain 4 h post-blood-feeding in 3-day-old females using dose response curves. Hunt et al. (2005) showed that pyrethroid-induced mortality was significantly reduced in 14- and 20-day-old blood-fed FUMOZ-R. These results collectively suggest that the expression of the resistance phenotype varies in response to fluctuations in physiological state, which is a function of various parameters including age, blood-feeding history and mating status. The increase in survival in females at 20 days of age warrants further investigation.

Specific primers were designed for the individual genes *CYP6P9* and *CYP6P13*. The effect of age on gene transcription in the resistant strain FUMOZ-R was investigated to test for a correlation between insecticide survival and gene transcription levels. Bioassay data showed that permethrin-induced mortality generally increased with age. In terms of gene transcription, the highest tCNR was observed in *CYP6P9* (9.30) and *CYP6P13* (4.82) at 10 days old. Permethrin survival of females was also highest between 3 and 10 days of age. However, statistical analysis revealed no significant overall correlation between gene transcription for either gene and susceptibility to permethrin. Further, the male tCNRs were higher than those of the females, in contrast to the bioassay data in which males showed a slightly lower survival than females. This discrepancy may be attributable to other biological functions (such as pheromone production) that P450s are associated with males (Kasai and Tomita, 2003).

 

WHO (1998) recommends that insecticide susceptibility be assessed using female mosquitoes aged 2-5 days. As a consequence, this age cohort has also been used to identify the underlying insecticide resistance mechanisms. However, females as young as this cannot be malaria infective and are therefore not representative of that portion of a vector population responsible for malaria transmission. This is the first study, to our knowledge, to investigate variation in resistance phenotypes at different ages looking for correlations with gene transcription in a highly resistant strain of *A. funestus* from southern Africa. The results show clearly that phenotype susceptibility increases with age and the P450 gene over-expression remains constant throughout the life span of the mosquitoes.

In conclusion, this study highlighted two important genes that have previously been shown to be associated with permethrin resistance in adults of southern African *A. funestus*. Both these genes showed transcription variation in association with mosquito age. However, transcript analysis provided only limited information as to how the function and effect of each enzyme varies with age. Future functional genomic studies will be necessary to clarify the role of these enzymes in pyrethroid metabolism. This is likely to prove complex as these enzymes may have multiple functions and their expression can be affected by numerous environmental as well as physiological factors. A recent analysis of field-collected *A. funestus* samples from Mozambique showed an association between pyrethroid resistance and the up-regulation of these two genes (Cuamba et al., 2010). However, this mode of resistance may not necessarily occur in other *A. funestus* populations.

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## REFERENCES

Amenya DA, Koekemoer LL, Vaughan A, Morgan JC, et al. (2005). Isolation and sequence analysis of P450 genes from a pyrethroid resistant colony of the major malaria vector *Anopheles funestus*. *DNA Seq.* 16: 437-445.

Amenya DA, Naguran R, Lo TC, Ranson H, et al. (2008). Over expression of a cytochrome P450 (CYP6P9) in a major African malaria vector, *Anopheles funestus*, resistant to pyrethroids. *Insect Mol. Biol.* 17: 19-25.

Bio-Rad (2006). Real-Time PCR Applications Guide. Bulletin 5279. Bio-Rad Laboratories, Inc., Hercules.

Brogdon WG and McAllister JC (1998). Insecticide resistance and vector control. *Emerg. Infect. Dis.* 4: 605-613.

Brooke BD, Kloke G, Hunt RH, Koekemoer LL, et al. (2001). Bioassay and biochemical analyses of insecticide resistance in southern African *Anopheles funestus* (Diptera: Culicidae). *Bull. Entomol. Res.* 91: 265-272.

Christian RN, Strode C, Ranson H and Coetzer N (2011). Microarray analysis of a pyrethroid resistant African malaria vector, *Anopheles funestus*, from southern Africa. *Pestic. Biochem. Physiol.* 99: 140-147.

Cuamba N, Morgan JC, Irving H, Steven A, et al. (2010). High level of pyrethroid resistance in an *Anopheles funestus* population of the Chokwe District in Mozambique. *PLoS One* 5: e11010.

Fontenille D, Lepers JP, Campbell GH, Coluzzi M, et al. (1990). Malaria transmission and vector biology in Manarintsoa, high plateaux of Madagascar. *Am. J. Trop. Med. Hyg.* 43: 107-115.

Gillies MT and De Meillon B (1968). The Anophelinae of Africa South of the Sahara. Publications of the South African Institute of Medical Research, Johannesburg.

Hargreaves K, Koekemoer LL, Brooke BD, Hunt RH, et al. (2000). *Anopheles funestus* resistant to pyrethroid insecticides in South Africa. *Med. Vet. Entomol.* 14: 181-189.

Hemingway J and Ranson H (2000). Insecticide resistance in insect vectors of human disease. *Annu. Rev. Entomol.* 45: 371-391.

Hodjati MH and Curtis CF (1999). Evaluation of the effect of mosquito age and prior exposure to insecticide on pyrethroid tolerance in *Anopheles mosquitoes* (Diptera: Culicidae). *Bull. Entomol. Res.* 89: 329-337.

Hunt RH, Brooke BD, Pillay C, Koekemoer LL, et al. (2005). Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector *Anopheles funestus*. *Med. Vet. Entomol.* 19: 271-275.

Kasai S and Tomita T (2003). Male specific expression of a cytochrome P450 (Cyp312a1) in *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* 300: 894-900.

Matambo TS (2008). Biochemical Characterisation of Cytochrome P450 Enzyme (CYP6P9) Involvement in Insecticide Resistance in the Major Malaria Vector *Anopheles funestus*. Doctoral thesis, University of Witwatersrand, Johannesburg.

Matambo TS, Paine MJ, Coetzee M and Koekemoer LL (2010). Sequence characterization of cytochrome P450 CYP6P9 in pyrethroid resistant and susceptible *Anopheles funestus* (Diptera: Culicidae). *Genet. Mol. Res.* 9: 554-564.

Morgan JC, Irving H, Okedi LM, Steven A, et al. (2010). Pyrethroid resistance in an *Anopheles funestus* population from Uganda. *PLoS One* 5: e11872.

Okoye PN (2008). Biology of Insecticide Resistance in the African malaria Vector *Anopheles funestus* (Diptera: Culicidae). Doctoral thesis, University of the Witwatersrand, Johannesburg.

Paton MG, Karunaratne SH, Giakoumaki E, Roberts N, et al. (2000). Quantitative analysis of gene amplification in insecticide-resistant *Culex* mosquitoes. *Biochem. J.* 346 Pt 1: 17-24.

Spillings BL, Coetzee M, Koekemoer LL and Brooke BD (2008). The effect of a single blood meal on the phenotypic expression of insecticide resistance in the major malaria vector *Anopheles funestus*. *Malar. J.* 7: 226.

WHO (1998). Test Procedures for Insecticide Resistance Monitoring in Malaria Vectors, Bio-Efficacy and Persistence of Insecticides on Treated Surfaces. Document WHO/CDS/CPC/MAL/98.12. World Health Organization, Geneva, Switzerland.

WHO (2008). World Malaria Report. Available at [http://apps.who.int/malaria/wmr2008/malaria2008.pdf]. Accessed December 18, 2011.

Wondji CS, Morgan J, Coetzee M, Hunt RH, et al. (2007). Mapping a quantitative trait locus (QTL) conferring pyrethroid resistance in the African malaria vector *Anopheles funestus*. *BMC Genomics* 8: 34.

Wondji CS, Irving H, Morgan J, Lobo NF, et al. (2009). Two duplicated P450 genes are associated with pyrethroid resistance in *Anopheles funestus*, a major malaria vector. *Genome Res.* 19: 452-459.