Cytochrome P450 metabolic resistance (CYP6P9a) to pyrethroids imposes a fitness cost in the major African malaria vector *Anopheles funestus*

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Abstract

Metabolic resistance threatens the sustainability of pyrethroid-based malaria control interventions. Elucidating the fitness cost and potential reversal of metabolic resistance is crucial to design suitable resistance management strategies. Here, we deciphered the fitness cost associated with the *CYP6P9a* (P450-mediated metabolic resistance) in the major African malaria vector *Anopheles funestus*. Reciprocal crosses were performed between a pyrethroid susceptible (FANG) and resistant (FUMOS-R) laboratory strains and the hybrid strains showed intermediate resistance. Genotyping the *CYP6P9a*-R resistance allele in oviposited females revealed that *CYP6P9a* negatively impacts the fecundity as homozygote susceptible mosquitoes (*CYP6P9a*-SS) lay more eggs than heterozygote (OR = 2.04; $P = 0.01$) and homozygote resistant mosquitoes. *CYP6P9a* also imposes a significant fitness cost on the larval development as homozygote resistant larvae (*CYP6P9a*-RR) developed significantly slower than heterozygote and homozygote susceptible mosquitoes ($\chi^2 = 11.2$; $P = 0.0008$). This fitness cost was further supported by the late pupation of homozygote resistant than susceptible mosquitoes (OR = 2.50; $P < 0.01$). However, *CYP6P9a* does not impact the longevity as no difference was observed in the life span of mosquitoes with different genotypes ($\chi^2 = 1.6$; $P = 0.9$). In this hybrid strain, a significant decrease of the resistant *CYP6P9a*-RR genotype was observed after ten generations ($\chi^2 = 6.6$; $P = 0.01$) suggesting a reversal of P450-based resistance in the absence of selection. This study shows that the P450-mediated metabolic resistance imposes a high fitness cost in malaria vectors supporting that a resistance management strategy based on rotation could help mitigate the impact of such resistance.

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Background

Malaria control relies mainly on insecticide-based interventions, notably pyrethroid-based long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS). Significant efforts have been made globally to eliminate malaria leading to consistent reduction in malaria cases and mortality in Africa by 42% and 66%, respectively (Bhatt et al. 2015; Riveron et al. 2018). However, increasing insecticide

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resistance in malaria vector species presents a major challenge to these vector control interventions and likely contributed to the increase in malaria incidence in the last 2 years (WHO 2018). To sustain the effectiveness of these interventions it is imperative to implement suitable insecticide resistance management (IRM) strategies to reduce the negative impact of such resistance. IRM strategies such as rotation of insecticide classes, rely on resistance alleles having a fitness cost inducing a selection against resistance alleles in the absence of insecticide selection pressure. Therefore, understanding the fitness cost that selection can act against mosquitoes is a key pre-requisite to effective IRM. Since pyrethroids are by far the most widely used insecticide class and the main one recommended for the impregnation of bed nets, elucidating the fitness costs of molecular mechanisms conferring pyrethroid resistance in mosquitoes could guide suitable control measures for malaria prevention.

The two main mechanisms driving pyrethroid resistance are target-site resistance (e.g., knockdown resistance, *kdr*) and metabolic resistance through over-expression of detoxification enzymes (e.g., cytochrome P450s, glutathione S-transferases and esterases) (Ranson et al. 2011; Riveron et al. 2018). Target-site resistance through *kdr* is well characterised, and the DNA-based diagnostic tools, were designed in the late 1990s (Martinez-Torres et al. 1998; Ranson et al. 2000). This allowed studying the fitness cost of target-site resistance on different life traits in a range of mosquito species including their reproduction, developmental time of immature stages, adult longevity and vector competence (Alout et al. 2016; Alout et al. 2014; Assogba et al. 2015; Brito et al. 2013; Martins et al. 2012). These studies highlighted that resistant vectors may have lower mating success, lower fecundity and fertility, higher developmental time and reduced longevity. The presence of such fitness costs that can impact the spread and persistence of resistance alleles in the vector populations is a pre-requisite for the implementation of most IRM strategies including rotation of insecticides. In contrast, metabolic resistance, a very common resistance mechanism in mosquitoes and considered to be more likely to cause control failure (Hemingway 2014), still had no molecular diagnostic tools, despite progress made in elucidating its molecular basis (Edi et al. 2014; Ibrahim et al. 2015; Mitchell et al. 2012; Riveron et al. 2013). This has prevented assessment of the fitness cost associated with this resistance mechanism and consequently hampered the design of suitable resistance management strategy to control malaria vectors. However, recent progress has been made in detecting key markers of metabolic resistance in major malaria vectors including for glutathione S-transferase mediated resistance such as the L119F-GSTe2 marker in *Anopheles funestus* (Riveron et al. 2014) and the I114T-

GSTe2 in *An. gambiae* (Mitchell et al. 2012). The design of the L119F-GSTe2 diagnostic tool recently allowed assessment of the fitness cost of GST-based metabolic resistance revealing significant cost in the GST-resistant mosquitoes, although also revealing that they live longer (Tchouakui et al. 2018) and are more infected with *Plasmodium* (Tchouakui et al. 2019). Recently, major progress was also made in detecting molecular marker for cytochrome P450-based resistance with the detection of *cis*-regulatory variants driving the expression of the major pyrethroid resistance gene *CYP6P9a* in *An. funestus* (Weedall et al. 2019). The simple PCR-RFLP assay designed has already helped to demonstrate that pyrethroid resistance is reducing the efficacy of LLINs as resistant mosquitoes were shown to significantly survive exposure to these nets and also blood fed more than the susceptible ones (Weedall et al. 2019). This diagnostic tool also provides an opportunity to assess the fitness cost of P450-mediated metabolic resistance in malaria vectors. *An. funestus* is particularly a suitable vector for assessing the impact of metabolic resistance as this mechanism is the main cause of pyrethroid resistance with the absence of *kdr* mutations consistently reported in populations of this malaria vector throughout Africa (Amenya et al. 2008; Okoye et al. 2008; Wondji et al. 2011; Riveron et al. 2013; Irving and Wondji 2017). One of the limitation of the studies on fitness cost is the use of resistant and susceptible strains from different geographical origins as they may differ in many other genes than those involved in resistance. For this reason, to minimise the effect of the genetic background on related fitness parameters, we proceeded by a crossing between the resistant and the susceptible strains so that the genetic background of the resistant strain is shared with that of the susceptible one as described elsewhere (Amin and White 1984; Argentine et al. 1989).

We elucidated the fitness cost of P450-based metabolic resistance on life traits of the malaria vector *An. funestus*, to better inform the design of suitable resistance management strategies against malaria vectors. This revealed a significant negative impact on fecundity and larval development of resistant mosquitoes *CYP6P9a-RR* while demonstrating a significant reduction of the frequency of resistant allele in the absence of selection.

Methods

Establishment of the mosquito strains

Reciprocal crosses were performed in January 2017 between FANG and FUM0Z-R, two *An. funestus* laboratory strains for several generations in order to bring the *CYP6P9a* resistance into a susceptible genetic background.

FUMOS-R is a pyrethroid (permethrin) resistant selected strain originates from southern Mozambique (FUMOS-R) (Hunt et al. 2005) and kept in colony since July 2001. This strain was selected based on its resistance status after 1 h exposure to permethrin after WHO bioassays (WHO 1998) and currently exhibits 0% mortality at 0.75% permethrin exposure. The FANG strain originates from southern Angola and kept in colony since January 2003 and is fully susceptible to all major vector control insecticides. Previous studies have shown that the *CYP6P9a* alleles conferring resistance in southern Africa is fixed in the FUMOS-R strain, whereas it is absent in FANG (Weedall et al. 2019). To perform the crossing, pupae of each strain were collected and put individually in 15 ml falcon tubes for individual emergence then, the males of the resistant strain were mixed into a same cage with the females of the susceptible colony (and reciprocally) for random mating to generate the first generation.

Mosquito's rearing

In each generation, eggs obtained from the crosses between both strains were transferred in paper cups containing mineral water for hatching. These eggs were flushed each day with mineral water and 2–3 days post-hatching, larvae obtained were transferred in larvae bowl and reared in mineral water with Tetramin® baby fish food everyday as described previously (Morgan et al. 2010). Water of each larvae bowl was changed every 2 days. The F_1 adults generated were randomly mixed in cages and fed with 10% sugar solution for crossing and production of the next generation. In each generation, after emergence of the adults, mosquitoes were let to randomly mate in cages for 5 days and blood fed three times before been allowed to lay eggs for the next generation. After the initial F_1 generation obtained from the reciprocal crosses, the hybrid strain FANG/FUMOS-R (hybrid stain from female FANG and males FUMOS-R) was reared till F_{10} generation in order to assess the fitness cost and a potential reversal of resistance.

Susceptibility profile of the hybrid FANG/FUMOS-R strain and validation of the implication of *CYP6P9a* in the resistance

WHO bioassays were carried out to assess the susceptibility profile of the two reciprocal hybrid strains for pyrethroids (0.75% permethrin and 0.05% deltamethrin), DDT (4%) and the carbamate bendiocarb (0.1%). The bioassays were performed according to WHO protocol (WHO 2013). In order to investigate the correlation between the *CYP6P9a* marker and pyrethroid resistance, additional bioassays were conducted with permethrin and deltamethrin for 30 min and 90 min. Alive mosquitoes after 90 min of exposure and

those dead after 30 min of exposition were then genotyped to establish the association between the *CYP6P9a-R* resistant allele and the ability of mosquitoes to survive to these insecticides.

Life traits experiments

All parameters were evaluated by simultaneously comparing fitness parameters between homozygotes resistant (*CYP6P9a-RR*), heterozygotes (*CYP6P9a-RS*) and homozygote susceptible (*CYP6P9a-SS*), reared together in the same containers and under the same environmental conditions such as larval density and feeding, temperature and light cycle.

Fecundity and fertility

In the 4th generation (F_4), after 4–5 days of mating in cages, females were blood fed three times and given 4 days to become fully gravid. Fully gravid-females were put individually in 1.5 ml Eppendorf tubes with damp filter paper to enable them to lay eggs as previously described (Morgan et al. 2010). The number of eggs laid per female and the number of larvae obtained after hatching were recorded. As a Shapiro–Wilk normality test showed non-normal distribution of eggs, the impact of resistance on fecundity was assessed by comparing the median number of eggs laid by different genotypes using a Kruskal–Wallis non-parametric test. In addition, odds ratio for oviposition between *CYP6P9a-RR*, *CYP6P9a-SS* and *CYP6P9a-RS* was also assessed using a statistical significance calculation based on the Fisher's exact probability test. The impact of resistance on fertility was assessed by comparing the hatch rate between the *CYP6P9a* genotypes using a Chi square test.

Larval and pupal development

After recording the total number of larvae produced per female, all larvae comprising the three *CYP6P9a* genotypes were pooled and reared together in the same larvae bowl, thus avoiding variation in environmental conditions. This experiment was performed in three replicates of ten trays per replicate and all immature stages were reared in the standard insectary condition. In order to prevent overcrowding and competition for food, larval bowls used were large enough to accommodate all larvae. The number of larvae varied between 200 and 300 per tray and water was changed every 2 days in each tray to minimise the effect of pollution from the food according to Morgan et al. (2010). Changes in the time of development of immature stages and mortality rates was equally assessed by genotyping about 100 larvae at different stages (L1, L2, L3 and L4). For this purpose, genotype frequency was monitored in each stage

of development. The dynamic of pupae formation was evaluated by comparing the genotype and allele frequencies from the first day of pupation (pupae day 9), in the third day of pupation (pupae day 11) and in the fifth day of pupation (pupae day 13). Chi-squared and odds ratio with Fisher's exact test were used to assess significance of the difference in genotype distribution between larval stages and pupae obtained at different time-points.

Adult longevity

After emergence of adults, a set of about 150 mosquitoes was removed from the cages at different time-points (day 1, 10, 20 and 30 after emergence). On average, 100 mosquitoes were used for genotyping whereas three pools of ten mosquitoes each were used to assess the gene expression level of *CYP6P9a* at each time-point. The life span of homozygous resistant adult mosquitoes was compared with that of susceptible and heterozygotes (RS) mosquitoes by assessing the frequency of *CYP6P9a* genotypes/alleles and the expression level of *CYP6P9a* (qRT-PCR) at different time-points.

Population cage experiments to assess a potential reversal to susceptibility

The dynamics of *CYP6P9a*-R resistant allele frequency in the absence of insecticide pressure was assessed using cage experiments. After crossing between female FANG and male FUMOS-R, the progeny obtained were let in cages for intercrosses for ten generations. In each generation, all mosquitoes irrespective of their genotypes were randomly mixed in cages for intercrossing to generate the next generation. Each generation consisted in about three cages of at least 200 mosquitoes/cage of mixed genotypes. In the first generation, the frequency of the *CYP6P9a*-R resistant allele was assessed and then monitored in following generations by genotyping a set of about 75 females aged between 2 and 5 days old.

Genotyping of the *CYP6P9a* resistance allele using PCR-RFLP

Genomic DNA was extracted from adult mosquitoes and all larval and pupal stages using the Livak method (Livak 1984). The genotyping of *CYP6P9a* resistance allele was done using PCR-RFLP method as recently described (Weedall et al. 2019). The RFLP6P9aF forward primer, 5'-TCC CGA AAT ACA GCC TTT CAG-3 and RFLP6P9aR 5'-ATT GGT GCC ATC GCT AGA AG-3' reverse primers are used in the first step amplification of the partial *CYP6P9a* upstream region. PCR reactions were carried out on genomic DNA from individual mosquitoes. The final

15 µl PCR mixture contained 1.5 µl of 10X KAPA Taq buffer A (KAPA Biosystems), 0.12 µl of 5 U/µl KAPA Taq polymerase, 0.12 µl of 25 µM dNTP, 0.75 µl of 25 µM MgCl₂, 0.51 µl of each primer, 10.49 µl of dH₂O, and 1 µl of genomic DNA. The PCR parameters were 95 °C for 5 min and 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s, followed by a final extension step of 72 °C for 10 min. The size of PCR products was obtained on 1.5% agarose gel stained with Midori Green Advance DNA Stain (Nippon genetics Europe GmbH, Dueren, Germany) and visualised using a gel imaging system to confirm the product size (450 bp). For the second step, the TaqI enzyme (restriction site (5'-TCGA-3')) was used to digest the PCR product and detect the *CYP6P9a*-R resistant allele as previously described (Weedall et al. 2019). For this second step, 10 µl of the digestion mix is made using 1 µl of CutSmart buffer, 0.2 µl of 2 units of TaqI restriction enzyme (New England Biolabs, Ipswich, MA, USA), 5 µl of PCR product, and 3.8 µl of dH₂O. The mix was incubated at 65 °C for 2 h and the product separated on 2.0% agarose gel stained with Midori Green. After this second step, *CYP6P9a*-RR displays one band at 350 bp, the *CYP6P9a*-SS showed one band as well at 450 bp whereas heterozygotes individuals present both bands.

Expression profile of *CYP6P9a* and adult longevity using qRT-PCR

The quantitative reverse-transcription PCR (qRT-PCR) was performed to assess the expression level of *CYP6P9a* from day 1 to day 30. Total RNA from three biological replicates (ten mosquitoes each) from day 1, day 10, day 20 and day 30 after adult emergence was extracted using the Picopure RNA Isolation Kit (Arcturus). One micrograms of RNA from each of the three biological replicates at each time-point, and FANG (full susceptible strain) was used as a template for cDNA synthesis using the superscript III (Invitrogen) with oligo-dT20 and RNase H, following the manufacturer's instructions. The qRT-PCR was carried out in a MX3005 real-time PCR system (Agilent) using Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent). A total of 10 ng of cDNA from each sample was used as template in a three-step programme involving a denaturation at 95 °C for 3 min followed by 40 cycles of 10 s at 95 °C and 10 s at 60 °C and a last step of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C as previously described (Kwiatkowska et al. 2013; Riveron et al. 2013). The relative expression level and fold-change (FC) of *CYP6P9a* in each time-point relative to the susceptible strain was calculated according to the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008) after normalisation with the housekeeping genes ribosomal protein S7 (RSP7; AFUN007153) and actin 5C (AFUN006819).

Results

Susceptibility profiles of the FUM0Z-R/FANG and FANG/FUM0Z-R strains

Bioassays conducted on the F_4 mosquitoes from the reciprocal crosses between females FANG/males FUM0Z-R (FANG/FUM0Z-R) and females FUM0Z-R/males FANG (FUM0Z-R/FANG) strains revealed that both hybrid strains were resistant to pyrethroids and carbamates as previously described by Weedall et al. (2019). Both strains were moderately resistant to DDT (93% mortality). For deltamethrin, a higher mortality rate was recorded for the strain generated from the crossing between females FUM0Z_R and males FANG (48.5% mortality) compared with the strain obtained from females FANG and males FUM0Z_R (77.3%) (Fig. S1). For bendiocarb (carbamate), the resistance pattern was similar in both reciprocal strains (Weedall et al. 2019).

Validation of the role of the *CYP6P9a* metabolic resistance in the observed pyrethroid resistance

Assessment of the differential expression of *CYP6P9a* in the hybrid strain after exposure to permethrin and deltamethrin revealed that the expression level of this gene was as followed: permethrin alive: $FC = 16.6 \pm 4.7$, $P = 0.01$; deltamethrin alive: $FC = 8.8 \pm 4.7$, $P = 0.04$ and unexposed mosquitoes $FC = 7.9 \pm 2.12$, $P = 0.01$. For both groups, this level of expression was significantly high compared with the susceptible strain FANG supporting that *CYP6P9a* plays a role in the resistance observed (Fig. 1b). Furthermore, the level of expression did not differ significantly between exposed and unexposed mosquitoes (Fig. 1a) showing that the gene is constitutively expressed in this line. To validate the role of the recently discovered CYP6P9a_R

resistant allele in the observed pyrethroid resistance we assessed the correlation between this allele and the ability of mosquitoes to survive after exposure. Mortality rates of 39.0% and 42.3% after 3-min exposure and mortality of 81.3% and 86.3% after 90-min exposure were obtained for permethrin and deltamethrin, respectively (Fig. 1c). Genotyping of the dead mosquitoes after 30-min exposure and the alive after 90-min exposure to these insecticides as previously described showed that the ability of homozygotes resistant mosquitoes to survive after exposition was higher compared with the homozygous susceptible (SS) as recently also described (Weedall et al. 2019).

Influence of the CYP6P9a_R on the fecundity/fertility of female mosquitoes

No significant difference was observed in the distribution of genotypes between females which have successfully laid eggs after blood feeding and those which did not laid eggs ($\chi^2 = 4.3$; $df = 2$; $p > 0.1$). However, a higher but not significant ($\chi^2 = 1.65$; $p = 0.19$) frequency of CYP6P9a_R resistant allele was observed in non-oviposited females (39%) compared with the oviposited females (33%). Assessment of the odd ratio (OR) for oviposition between homozygote resistant (RR) mosquitoes, SS and RS mosquitoes using Fisher's exact probability test revealed that the ability of SS mosquitoes to lay eggs was higher compared with RS (OR = 2.04; confidence interval (CI) 95%: 1.1–3.8; $p = 0.01$). The same trend was observed when compared with RR although not significant (OR = 2.0; CI 95%: 0.7–5.7; $p = 0.15$) (Fig. 2a, b) suggesting that mosquitoes harbouring the resistant allele have less chance to lay eggs compared with those with the susceptible allele. RS mosquitoes displayed the same ability of oviposition than RR (OR = 1; CI 95%: 0.4–2.6; $p = 0.57$)

Fig. 1 Validation of the implication of *CYP6P9a* in the resistance to pyrethroids. **a** Differential expression by quantitative reverse-transcription polymerase chain reaction of *CYP6P9a* in the hybrid strain after exposition to permethrin and deltamethrin compared with the susceptible strain FANG; An asterisk indicates significant high differential expression of *CYP6P9a* gene in comparison with

susceptible strain, NS not significant. **b** Distribution of the *CYP6P9a* genotypes according to resistance phenotypes. FANG/FUM0Z-R represents a line obtained from the crossing between female FANG and male FUM0Z-R whereas FUM0Z-R/FANG represents a line obtained from the crossing between female FUM0Z-R and male FANG; Perm permethrin, Delt deltamethrin.

Fig. 2 Fecundity and fertility of females with different genotypes of the *CYP6P9a* gene. **a, b** Schematic representation of the impact of *CYP6P9a* genotypes on egg-laying success with odd ratio (OR). **c** Number of eggs laid by the *CYP6P9a*-RR, *CYP6P9a*-RS and *CYP6P9a*-SS genotypes. **d** Number of larvae generated by females from each genotype. **e** Comparison of the mean number of eggs laid and hatching rate between the three genotypes. Median value with

interquartile range is shown for each distribution. Dotted line indicates females for which at least 50 eggs or larvae were obtained. Difference between genotypes was not significant in term of eggs laying by Kruskal–Wallis non-parametric test whereas the number of larvae produced, and the hatch rate differed significantly. ***Significant difference at $p < 0.001$; *significant difference at $P < 0.05$; NS not significant.

Table 1 Assessment of the association between *CYP6P9a* genotypes and the ability of females to lay eggs.

Combination of genotypes at the <i>CYP6P9a</i> locus	Level of association	
	Odds ratio	<i>p</i> value
SS vs. RR	2.0 (0.7–5.7)	0.15
SS vs. RS	2.04 (1.1–3.8)	0.01*
RS vs. RR	1 (0.4–2.6)	0.57
S vs. R	1.4 (0.8–2.6)	0.1

SS homozygote susceptible, RR homozygote resistant, RS heterozygote.

An asterisk indicates significant difference $p < 0.05$.

(Table 1 and Fig. 2b) suggesting a non-additional burden of the *CYP6P9a_R* allele on fecundity.

Furthermore, the median number of eggs laid per female for *CYP6P9a_RR* was 53.7 ± 10.05 (min = 7; max = 124). The median was 69.58 ± 5.04 (ranged from 4 to 137) for *CYP6P9a_RS* while *CYP6P9a_SS* laid 74.52 ± 4.06 eggs per female (ranged from 8 to 185) (Fig. 2c). However, a Kruskal–Wallis non-parametric test showed no statistical

difference ($p = 0.2$) (Fig. 2e). Concerning the viability of eggs laid, RR mosquitoes produced 28.0 ± 10.31 larvae (min = 0; max = 98) corresponding to a hatch rate of $39 \pm 11.86\%$. For RS mosquitoes the mean number of larvae was 43.7 ± 4.9 larvae (min = 0; max = 125) corresponding to a hatch rate of $56.1 \pm 4.0\%$ while SS mosquitoes produced 52.6 ± 4.0 larvae (min = 0; max = 118) corresponding to a hatch rate of $67.0 \pm 3.9\%$ (Fig. 2d). The mean number of larvae was lower in RR ($P = 0.02$) compared with other genotypes as well as for the hatch rate ($p < 0.04$) (Fig. 2e).

Level of association between the *CYP6P9a_R* resistant allele and larval development

Egg-hatching occurred 2 days post-oviposition and development time from the larvae to the pupae was 12.5 ± 4.5 days overall (Fig. 3). Genotyping of 100 randomly collected larvae for each L1, L2, L3 and L4 stages at F_8 generation revealed a significant and consistent decrease of the resistant allele *CYP6P9a_R* from L1 to L4, indicating greater mortality or slower development of the resistant mosquitoes during this immature stage. A reduction of RR

Fig. 3 Distribution of the *CYP6P9a* genotypes at different time-points of the development of immature stages. **a** Stacked bar plot of the variation in genotype frequency during the development of larvae (L1, L2, L3, and L4 represent different larval stages) and pupae formation (**b**); **c** the proportion of larvae surviving at each developmental

stage from hatching (day 1) to formation of the pupae; **d** the proportion of pupae obtained in day 9, day 11 and day 13 of development. Coloured bars and lines indicate respectively *CYP6P9a*-RR, *CYP6P9a*-RS and *CYP6P9a*-SS genotypes.

Fig. 4 Influence of *CYP6P9a* on the adult longevity of *An. funestus*. Distribution of *CYP6P9a* genotypes (**a**) and alleles (**b**) at different time in the survived mosquitoes; dotted line indicates a frequency of 50% for the resistant and susceptible alleles. **c** Differential expression by quantitative reverse-transcription polymerase chain reaction of *CYP6P9a* genes in alive mosquitoes at different time-points compared with the susceptible lab strain FANG. Error bars represent standard error of the mean; An asterisk indicates statistically significant differential expression of the gene in comparison with susceptible strain; NS not significant.

was observed from L1 (11%) to L4 (5%) although this was not significant, possibly due to the low number of RR ($\chi^2 = 1.7$; $P = 0.2$) (Fig. 4a, b). A statistically significant decrease

of the RS genotype was consistently observed from L1 (49%) to L4 (27%) ($\chi^2 = 7.2$; $P = 0.007$) together with a significant increase of the homozygote susceptible genotype

Table 2 Association between *CYP6P9a* genotypes/alleles and pupae formation.

Combination of genotypes at the <i>CYP6P9a</i> locus	Day 9, Day 11		Day 9, Day 13		Day 11, Day 13	
	Odds ratio	<i>p</i> value	Odds ratio	<i>p</i> value	Odds ratio	<i>p</i> value
SS vs. RR	3.0 (0.5–16.3)	0.2	7.5 (1.5–34.4)	0.007*	2.5 (0.6–1.9)	0.1
SS vs. RS	1.2 (0.9–2.2)	0.08	2.03 (0.9–4.9)	0.08	1.7 (0.7–3.9)	0.2
RS vs. RR	2.5 (0.5–14.3)	0.3	3.7 (0.7–18.7)	0.09	1.4 (0.5–4.5)	0.5
S vs. R	1.5 (0.8–2.8)	0.1	2.5 (1.4–4.6)	0.002*	1.7 (0.9–3.0)	0.05*

An asterisk indicates significant difference.

Fig. 5 Evaluation of the reversal to susceptibility in the hybrid colony FANG/FUMOZ-R. Changes in the *CYP6P9a* genotypes (a) and allele (b) for ten generations in the insecticides free environment.

SS from L1 (40%) to L4 (68%) ($\chi^2 = 12.15$; $P = 0.0004$) supporting a significant fitness cost of *CYP6P9a* on the larval development of resistant mosquitoes.

Pupae were obtained from 9 days post-hatching (pupae day 9) to 17 days (pupae day 17) with most pupation (more than 75%) observed at 11 days post-hatching (pupae day 11). Assessment of the rate of pupae formation by comparing the frequency of the *CYP6P9a* genotypes in the pupae obtained in day 9, day 11 and day 13 showed a consistent decrease of the homozygote susceptible SS genotype from day 9 (58%) to day 11 (34%) ($\chi^2 = 1.73$; $P = 0.19$) together with a significant increase of the RR genotype and RS from day 9 to day 13 ($\chi^2 = 11.17$; $P = 0.0008$) confirming that homozygote susceptible mosquitoes developed significantly faster than RR and RS mosquitoes (Fig. 4c, d). Assessment of the OR for pupae formation further supported that *CYP6P9a*-SS mosquitoes developed significantly faster than *CYP6P9a*-RR (OR = 2.50; $p < 0.01$) whereas there was no difference with *CYP6P9a*-RS (OR = 1.18; $p < 0.6$) (Table 2).

Assessment of the association between *CYP6P9a*-R allele and adult longevity

In average 100 alive mosquitoes were genotyped at day 1, day 10, day 20 and day 30 after the adult emergence to assess the association between the *CYP6P9a*-R allele and

F represents each generation; dotted line indicates a frequency of 50% for the resistant and susceptible alleles.

adult longevity. Comparison of genotypes frequency showed no difference in the distribution of genotypes ($\chi^2 = 1.6$; $p = 0.9$) (Fig. 4a) and alleles ($\chi^2 = 0.65$; $p = 0.88$) from day 1 to day 30 (Fig. 4b). In addition, assessment of the OR showed no difference in the life span of SS compared with RR (OR < 1.1; $p > 0.4$) and RS (OR < 1.1; $p > 0.2$) (Table S1). Evaluation of the expression level of *CYP6P9a* at the same time-points showed no significant difference of the level of expression of this gene in day 1 (fold-change (FC) = 14.03 ± 3.50), day 10 (FC = 13.4 ± 7.2), day 20 (FC = 13.7 ± 4.6), day 30 (FC = 9.2 ± 3.8) ($F = 1.08$; $df = 3$; $p = 0.4$) suggesting that over-expression of this P450 gene is not affecting the longevity of female mosquitoes (Fig. 4c).

Assessment of the reversal to susceptibility

The fitness cost of the *CYP6P9a*-R was also investigated in cage experiments to assess a potential reversal to susceptibility by examining the changes in the frequency of this allele over ten generations in the absence of insecticide selection pressure. A frequency of 50% of the resistant allele was confirmed in the F₁ generation of the FANG/FUMOZ-R as well as a 50% for the susceptible allele. A significant and consistent increase in the proportion of homozygote susceptible mosquitoes was observed from F₂ (20%) to F₁₀ (54%) ($\chi^2 = 6.2$; $P = 0.01$) (Fig. 5a) suggesting a reversal to susceptibility. This was supported by

an increase in the frequency of the susceptible allele from F_1 (50%) to F_{10} (70%) ($\chi^2 = 4.3$; $P = 0.03$) (Fig. 5b and Table S2).

Discussion

In this study, using the recently designed diagnostic assay for *CYP6P9a* gene, we investigated the fitness cost associated with P450-based resistance on various life traits of malaria vectors using laboratory strains of *An. funestus* revealing a significant cost imposed by P450-mediated resistance.

Association between the *CYP6P9a*-based resistance and fecundity/fertility

The results obtained in this study suggest that *CYP6P9a* induces a reduction in mosquitoes' fecundity and fertility. Same observation of reduced fecundity caused by metabolic based resistance was recently reported for the L119F-GSTe2 marker (Tchouakui et al. 2018). This reduced performance of RR mosquitoes in laying eggs could be associated to a reduction in the rate of insemination of resistant mosquitoes compared with the susceptible as previously reported (Brito et al. 2013). Several studies have previously reported a reduction in the number of eggs laid by insecticide resistant strains when compared with the susceptible strain (Brewer and Trumble 1991; Bouvier et al. 2001). In this study, homozygotes resistant mosquitoes displayed a significant lower viability of eggs compared with other genotypes as observed in *Ae. aegypti* (Mebrahtu et al. 1997).

Effect of the *CYP6P9a*-R resistant allele on the developmental time of the larvae

The developmental time of the larvae is a primary aspect of fitness in disseminating mosquito populations (Charlesworth 1994) as the survival rate of larvae or pupae might be reduced in the presence of natural predators or parasites (Agnew and Koella 1999). We observed a greater mortality/slower development of the resistant mosquitoes during larval development compared with the susceptible ones. This is a clear evidence of fitness cost imposed by P450-based metabolic resistance in mosquitoes. A range of environmental factors can affect the developmental time and survivorship of larvae and pupae including temperature, nutrition and larval density (Lyimo et al. 1992; Reisen 1995). In our study, temperature was controlled (25 ± 2 °C), nutrition and larval density as well. In addition, mosquitoes of the three genotypes were maintained together in the same containers thus limiting all the confounding factors from

environmental conditions. A greater mortality associated with a slower development of mosquitoes with the *CYP6P9a*-R resistant allele could be linked to the competition for food. Probably, despite the fact that all the three genotypes were maintained in the same larval bowl, larvae with *CYP6P9a*-R resistant allele were less competitive for food and space compared with those with the susceptible allele and therefore, developed significantly slower. As observed previously in resistant *Culex pipiens* for carboxylesterase-mediated metabolic resistance (Foster et al. 2003), the over-expression of *CYP6P9a* is probably linked with a decreased locomotive performance limiting the ability of mosquitoes with the resistant allele to move faster to feed. All this together could explain the longer developmental time observed in *CYP6P9a*-RR mosquitoes compared with RSs *CYP6P9a*-RS and *CYP6P9a*-SS susceptible mosquitoes. A similar high fitness cost was previously reported for target-site resistance such as *kdr* in the dengue vector *Aedes aegypti* (Brito et al. 2013) but this study provides the first evidence that cytochrome P450-based metabolic resistance induces a significant fitness cost on larval development in malaria vectors. The high fitness cost of the *CYP6P9a*-R resistance allele on the larval mortality and/or time of development of immature stages of resistant mosquitoes suggests that a resistance management strategy implemented before the allele becomes fixed in the population could effectively reduce P450-mediated metabolic resistance in the field. In contrast, a recent study on the impact of GST-based metabolic resistance on larval development found a RS advantage in term of developmental time compared with RR and susceptible mosquitoes for the L119F-GSTe2 marker (Tchouakui et al. 2018). This indicates that the cost of metabolic resistance on the physiological traits of mosquitoes can vary from one enzyme family to another and highlights the necessity to avoid extrapolation and to analyse such fitness cost in more metabolic resistance genes.

Association between the *CYP6P9a* resistance marker and female longevity

In contrast with other life traits, there was no association between the *CYP6P9a*-R resistant allele and adult longevity. Although this need to be assessed in field condition, this observation may suggest that the impact of *CYP6P9a*-R on the vectorial capacity of resistant mosquitoes might be less pronounced than that observed for the L119F-GSTe2 mutation which was shown to increase the longevity of resistant mosquitoes (Tchouakui et al. 2018). Because the vectors have to live sufficiently longer to allow the parasite to develop until the infective stage (McCarroll and Hemingway 2002), longevity of adult vectors is a key life-trait for which a change due to fitness cost could impact the disease

transmission. Rivero et al. reported that P450 monooxygenases and the GSTs particularly could drastically alter ROS levels in insects, albeit in radically opposite ways (Rivero et al. 2010). GSTs are known to protect mosquitoes against oxidative stress which results in the increase longevity whereas the increased activity of monooxygenases is associated with increase oxidative stress in mosquitoes (de Montellano 2015). The increase oxidase stress due to overproduction of monooxygenases could therefore reduce the longevity of insects although no such impact was seen in this study. Further studies with field populations will help further assess the extent of the effect of *CYP6P9a* gene on the life span of resistant mosquitoes in natural conditions.

Monitoring the reversal to susceptibility

Reversion to susceptibility is expected if the resistant gene harbours a fitness cost in an insecticide-free environment. Therefore, once insecticide pressure ceases, the frequency of the resistant allele, and consequently insecticide resistance, will decrease because of the fitness cost of insecticide resistance on mosquito's life traits. Knowledge of the reversal rate for insecticides such as pyrethroids is therefore crucial before implementing any resistance management strategy in the field. In this study, significant decrease in the frequency of the *CYP6P9a*-R resistant allele was observed after ten generations in the insecticide-free environment, which correspond to around 1 year. As previously observed (Saavedra-Rodriguez et al. 2012), this reduction in the resistant allele frequency could be attributed either to the accumulation of deleterious effects observed in some life traits of the vector as noticed for fecundity and larval development here or to the pleiotropic effect of other genes very close to *CYP6P9a* such as *CYP6P9b* since this gene is duplicated in *An. funestus*. Mating, copulation and insemination efficiency are other key factors which were not assessed in this study but which could have contributed to the reversal observed since female anophelids are inseminated only once during their life span. In these latter cases, males must be able to compete for copula, as the first to inseminate the female will increase the chance of propagating its genes. In the mosquito *Culex pipiens* males from a susceptible strain showed an advantage when competing for mating compared with males bearing three distinct organophosphate resistant genotypes (Berticat et al. 2002). Similarly, *kdr* and *Rdl* resistant males *An. gambiae* were shown to exhibit a lower mating competitiveness than susceptible ones (Platt et al. 2015). If such reduced mating competitiveness is also observed for this *An. funestus* strain, this could have contributed to the reversal noticed here. Such reversal to susceptibility suggests that resistance management strategies such as insecticide rotation could help to reverse

CYP6P9a-mediated metabolic resistance if implemented early. However, reversal rates can vary and may be very slow or impossible, particularly when an insecticide has been used for several years. For example, in Sri Lanka, the extensive use of DDT for malaria control for about 20 years up to the 1960s selected a resistance in *An. culicifacies* s.l. and *An. subpictus*. For this reason, DDT was replaced by malathion in the early 1970s and DDT resistance reverted very slowly towards susceptibility from 80% resistance in the 1970s to about 50% in the 1990s (Corbel and N'Guessan 2013). The same pattern of result was obtained in West Africa where a reversion of the resistance was observed in Northern Nigeria 6 years after the discontinuation of dieldrin spraying in *An. gambiae* population (Hamon and Garrett-Jones 1963). In Northern Nigeria, after 20–24 month of massive use of dieldrin, RR mosquitoes was the only ones remaining in the *An. gambiae* population with only few RRs. But, after 6 months in the absence of dieldrin, the homozygote susceptible genotype took over. Similar results were reported for *An. culicifacies* in India (Bhatia and Deobhankar 1963). However, the same allele at the *rdl* locus has been reported to be maintained in field populations in Sri Lanka despite the withdrawal of cyclodiene insecticides for mosquito control for more than 30 years (Roush and McKenzie 1987). Altogether these variations indicate that knowledge of the reversal rate of an insecticide is crucial for implementing any resistance management strategy in the field based on rotation of insecticides.

Conclusion

This study has investigated the fitness cost of P450-based metabolic resistance to pyrethroids in a major malaria vector revealing significant fitness cost for fecundity, fertility and the larval development of resistant mosquitoes. This fitness cost was further supported by the observation of a return to susceptibility in the absence of insecticide over ten generations (around 1 year) showing that if suitable resistance management strategies such as rotation was implemented, P450-based resistance could be managed. This should encourage future strategies using non-pyrethroid-based LLINs to reduce the selection pressure and allow such rotation to slow the spread of pyrethroid resistance.

Data availability

Underlying data is available here DOI (<https://doi.org/10.5061/dryad.pnvx0k6j4>).

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Author contributions CSW conceived and designed the study; MaT carried out the sample collection; MaT, DD and WT reared and maintained the strain in the insectary; MaT, MiT, LMJM and MJW performed the molecular analyses; MaT, JMR and CSW analysed the data; MaT and CSW wrote the paper with contributions from FN. All authors read and approved the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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