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

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**Running Title**

Fitness costs of P450-mediated metabolic resistance

## 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 (FUMOZ-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 10 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.



**Keywords:** Malaria, mosquito, *Anopheles funestus,* insecticide resistance, cytochrome P450, fitness cost.

## 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 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 two 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 prerequisite 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. knock-down 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 knockdown resistance (*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 mosquitoes species including

51 their reproduction, developmental time of immature stages, adult longevity and vector
52 competence (Alout et al. 2016, Alout et al. 2014, Assogba et al. 2015, Brito et al. 2013,
53 Martins et al. 2012). These studies highlighted that resistant vectors may have lower mating
54 success, lower fecundity and fertility, higher developmental time and reduced longevity. The
55 presence of such fitness costs that can impact the spread and persistence of resistance alleles
56 in the vector populations is a pre-requisite for the implementation of most insecticide
57 resistance management (IRM) strategies including rotation of insecticides. In contrast,
58 metabolic resistance, a very common resistance mechanism in mosquitoes and considered to
59 be more likely to cause control failure (Hemingway 2014), still had no molecular diagnostic
60 tools, despite progress made in elucidating its molecular basis (Edi et al. 2014, Ibrahim et al.
61 2015, Mitchell et al. 2012, Riveron et al. 2013). This has prevented assessment of the fitness
62 cost associated with this resistance mechanism and consequently hampered the design of
63 suitable resistance management strategy to control malaria vectors. However, recent progress
64 has been made in detecting key markers of metabolic resistance in major malaria vectors
65 including for glutathione S-transferase mediated resistance such as the L119F-GSTe2 marker
66 in *An. funestus* (Riveron et al. 2014) and the I114T-GSTe2 in *An. gambiae* (Mitchell et al.
67 2019). The design of the L119F-GSTe2 diagnostic tool recently allowed assessment of the
68 fitness cost of GST-based metabolic resistance revealing significant cost in the GST-resistant
69 mosquitoes, although also revealing that they live longer (Tchouakui et al. 2018) and are more
70 infected with *Plasmodium* (Tchouakui et al. 2019). Recently, major progress was also made
71 in detecting molecular marker for cytochrome P450 based resistance with the detection of cis-
72 regulatory variants driving the expression of the major pyrethroid resistance gene *CYP6P9a* in
73 *An. funestus* (Weedall et al. 2019). The simple PCR-RFLP assay designed has already helped
74 to demonstrate that pyrethroid resistance is reducing the efficacy of LLINs as resistant
75 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 FUMOZ-R, two *Anopheles funestus* laboratory strains for several generations in order to bring the *CYP6P9a* resistance into a susceptible genetic background. FUMOZ-R is a pyrethroid (permethrin) resistant selected strain originates from southern Mozambique (FUMOZ-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 FUMOZ-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 15ml 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 every day as described previously (Morgan et al. 2010). Water of each larvae bowl was changed every two 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 five 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/FUMOZ-R (hybrid stain from female FANG and males FUMOZ-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/FUMOZ-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 30min and 90min. Alive mosquitoes after 90min of exposure and those dead after 30min 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 $4^{th}$ 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 1.5ml 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 two days in each tray to minimize 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 day9), in the third day of pupation (pupae day11) and in the fifth day of pupation (pupae day13). 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 3 pools of 10 mosquitoes each were used to assess the gene expression level of *CYP6P9a* at each time point. The lifespan of homozygous resistant adult mosquitoes was compared to that of susceptible and heterozygotes 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 FUMOZ-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 3 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 2-5days 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 15µl PCR mixture contained 1.5µl of 10X KAPA Taq buffer A (KAPA Biosystems), 0.12µl of 5 U/µl KAPA Taq polymerase, 0.12µl of 25µM dNTP, 0.75µl of 25µM MgCl2, 0.51µl of each primer, 10.49µl of dH2O and 1µl of genomic DNA. The PCR parameters were 95°C for 5 minutes and 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 45 seconds, followed by a final extension step of 72°C for 10 minutes. 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 (450bp). 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, 10µl of the digestion mix is made using 1µl of CutSmart buffer, 0.2µl of 2 units of TaqI restriction enzyme (New England Biolabs, Ipswich, MA, USA), 5µl of PCR product and 3.8µl of $dH_20$. The mix was incubated at 65°C for 2 hours and the product separated on 2.0% agarose gel stained with Midori Green. After this second step, CYP6P9a-RR displays one band at 350bp, the CYP6P9a-SS showed one band as well at 450bp 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 day1 to day 30. Total RNA from three biological replicates (ten mosquitoes each) from day 1, day 10, day20 and day30 after adult emergence was extracted using the Picopure RNA Isolation Kit (Arcturus). 1 mg 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 program 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 normalization with the housekeeping genes ribosomal protein S7 (RSP7; AFUN007153) and actin 5C (AFUN006819).

## Results

### Susceptibility profiles of the FUMOZ-R/FANG and FANG/FUMOZ-R strains

Bioassays conducted on the $F_4$ mosquitoes from the reciprocal crosses between females FANG/males FUMOZ-R (FANG/FUMOZ-R) and females FUMOZ-R/males FANG (FUMOZ-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 FUMOZ_R and males FANG (48.5% mortality) compared to the strain obtained from females FANG and males FUMOZ_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±4.7, *P* =0.01; deltamethrin alive: FC= 8.8 ± 4.7, *P* =0.04 and unexposed mosquitoes FC= 7.9±2.12, *P* =0.01. For both groups, this level of expression was significantly high compared to 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. A mortality rates of 39.0% and 42.3% after 30 minutes' exposure and mortality of 81.3% and 86.3% after 90 minutes' exposure, were obtained for permethrin and deltamethrin respectively (Fig. 1C). Genotyping of the dead mosquitoes after 30min exposure and the alive after 90min exposure to these insecticides as previously described showed that the ability of homozygotes resistant mosquitoes to survive after exposition was higher compared to 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 ($x^2$ = 4.3; df= 2; $p > 0.1$). However, a higher but not significant ($x^2 = 1.65$; $p = 0.19$) frequency of CYP6P9a_R resistant allele was observed in non-oviposited females (39%) compared to the oviposited females (33%). Assessment of the odd-ratio (OR) for oviposition between homozygote resistant mosquitoes (RR), homozygote susceptible (SS) and heterozygote mosquitoes (RS) using Fisher's exact probability test revealed that the ability of SS mosquitoes to lay eggs was higher compared to RS (OR = 2.04; confidence interval (CI) 95%: 1.1–3.8; *p* =0.01). The same trend was observed when compared to homozygote resistant

(RR) although not significant (OR = 2.0; CI 95%: 0.7–5.7; $p$ =0.15) (Fig. 2A-B) suggesting that mosquitoes harboring the resistant allele have less chance to lay eggs compared to 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) (Table 1, 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 Kruskall-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% ± 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% ± 4.0% while SS mosquitoes produced 52.6 ± 4.0 larvae (min = 0; max = 118) corresponding to a hatch rate of 67.0% ± 3.9% (Fig 2D). The mean number of larvae was lower in RR ($P$ = 0.02) compared to 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. 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 homozygote resistant RR was observed from L1 (11%) to L4 (5%) although this was not

301 significant, possibly due to the low number of RR ($\chi^2$= 1.7; *P*=0.2) (Fig. 4A-B). A statistically
302 significant decrease of the heterozygote RS genotype was consistently observed from L1
303 (49%) to L4 (27%) ($\chi^2$ = 7.2; *P* = 0.007) together with a significant increase of the
304 homozygote susceptible genotype SS from L1 (40%) to L4 (68%) ($\chi^2$ = 12.15; *P* = 0.0004)
305 supporting a significant fitness cost of *CYP6P9a* on the larval development of resistant
306 mosquitoes.

307 Pupae were obtained from 9 days post-hatching (pupae day9) to 17 days (pupae
308 day17) with most pupation (more than 75%) observed at 11 days post-hatching (pupae
309 day11). Assessment of the rate of pupae formation by comparing the frequency of the
310 *CYP6P9a* genotypes in the pupae obtained in day9, day11 and day13 showed a consistent
311 decrease of the homozygote susceptible SS genotype from day9 (58%) to day11 (34%) ($\chi^2$ =
312 1.73; *P* = 0.19) together with a significant increase of the homozygote resistant genotype RR
313 and heterozygote RS from day9 to day13 ($\chi^2$= 11.17; *P* = 0.0008) confirming that homozygote
314 susceptible mosquitoes developed significantly faster than homozygote resistant and
315 heterozygote mosquitoes (Fig. 4C-D). Assessment of the OR for pupae formation further
316 supported that CYP6P9a-SS mosquitoes developed significantly faster than CYP6P9a-RR
317 (OR = 2.50; p < 0.01) whereas there was no difference with CYP6P9a-RS (OR =1.18; p <
318 0.6) (Table 2).
319
320 **Assessment of the association between CYP6P9a-R allele and adult longevity**
321 In average 100 alive mosquitoes were genotyped at day1, day10, day20 and day30 after
322 the adult emergence to assess the association between the CYP6P9a-R allele and adult
323 longevity. Comparison of genotypes frequency showed no difference in the distribution of
324 genotypes ($\chi^2$ = 1.6; *p* = 0.9) (Fig. 4A) and alleles ($\chi^2$ = 0.65; *p* = 0.88) from day1 to day30
325 (Fig. 4B). In addition, assessment of the OR showed no difference in the life span of SS

compared to 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 day1 (fold-change (FC) = 14.03 ± 3.50), day10 (FC = 13.4 ± 7.2), day20 (FC = 13.7 ± 4.6), day30 (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 females 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 10 generations in the absence of insecticide selection pressure. A frequency of 50% of the resistant allele was confirmed in the $F_1$ 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 $F_2$ (20%) to $F_{10}$ (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; 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 homozygote resistant mosquitoes in laying eggs could be associated to a reduction in the rate of insemination of resistant mosquitoes compared to 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 to 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 to 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 to 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 to 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 homozygote resistant mosquitoes compared to heterozygotes 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 heterozygote advantage in term of developmental time compared to homozygote resistant 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 analyze 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

401 observation may suggest that the impact of CYP6P9a-R on the vectorial capacity of resistant
402 mosquitoes might be less pronounced than that observed for the L119F-GSTe2 mutation
403 which was shown to increase the longevity of resistant mosquitoes (Tchouakui et al. 2018).
404 Because the vectors have to live sufficiently longer to allow the parasite to develop until the
405 infective stage (McCarroll and Hemingway 2002), longevity of adult vectors is a key life-trait
406 for which a change due to fitness cost could impact the disease transmission. Rivero *et al*
407 reported that P450 monoxygenases and the GSTs particularly could drastically alter ROS
408 levels in insects, albeit in radically opposite ways (Rivero et al. 2010). GSTs are known to
409 protect mosquitoes against oxidative stress which results in the increase longevity whereas the
410 increased activity of monoxygenases is associated with increase oxidative stress in
411 mosquitoes (de Montellano and De Voss 2005). The increase oxidase stress due to
412 overproduction of monoxygenases could therefore reduce the longevity of insects although no
413 such impact was seen in this study. Further studies with field populations will help further
414 assess the extent of the effect of *CYP6P9a* gene on the lifespan of resistant mosquitoes in
415 natural conditions.
416
417 **Monitoring the reversal to susceptibility**
418 Reversion to susceptibility is expected if the resistant gene harbors a fitness cost in an
419 insecticide-free environment. Therefore, once insecticide pressure ceases, the frequency of the
420 resistant allele, and consequently insecticide resistance, will decrease because of the fitness
421 cost of insecticide resistance on mosquito's life traits. Knowledge of the reversal rate for
422 insecticides such as pyrethroids is therefore crucial before implementing any resistance
423 management strategy in the field. In this study, significant decrease in the frequency of the
424 CYP6P9a-R resistant allele was observed after ten generation in the insecticide free
425 environment, which correspond to around 1 year. As previously observed (Saavedra

426 Rodriguez et al. 2012), this reduction in the resistant allele frequency could be attributed
427 either to the accumulation of deleterious effects observed in some life traits of the vector as
428 noticed for fecundity and larval development here or to the pleiotropic effect of other genes
429 very close to *CYP6P9a* such as *CYP6P9b* since this gene is duplicated in *An. funestus* .
430 Mating, copulation and insemination efficiency are other key factors which were not assessed
431 in this study but which could have contributed to the reversal observed since females
432 anopheles are inseminated only once during their lifespan. In these latter cases, males must be
433 able to compete for copula, as the first to inseminate the female will increase the chance of
434 propagating its genes. In the mosquito *Culex pipiens* males from a susceptible strain showed
435 an advantage when competing for mating compared to males bearing three distinct
436 organophosphate resistant genotypes (Berticat et al. 2002). Similarly, *kdr and Rdl* resistant
437 males *An. gambiae* were shown to exhibit a lower mating competitiveness than susceptible
438 ones (Platt et al. 2015). If such reduced mating competitiveness is also observed for this *An.*
439 *funestus* strain, this could have contributed to the reversal noticed here. Such reversal to
440 susceptibility suggests that resistance management strategies such as insecticide rotation
441 could help to reverse CYP6P9a-mediated metabolic resistance if implemented early.
442 However, reversal rates can vary and may be very slow or impossible, particularly when an
443 insecticide has been used for several years. For example, in Sri Lanka, the extensive use of
444 DDT for malaria control for about 20 years up to the 1960s selected a resistance in *An.*
445 *culicifacies* s.l. and *An. subpictus*. For this reason, DDT was replaced by malathion in the
446 early 1970s and DDT resistance reverted very slowly towards susceptibility from 80%
447 resistance in the 1970s to about 50% in the 1990s (Corbel and N'Guessan 2013). The same
448 pattern of result was obtained in West Africa where a reversion of the resistance was observed
449 in Northern Nigeria six years after the discontinuation of dieldrin spraying in *An. gambiae*
450 population (Hamon and Garrett-Jones 1963). In Northern Nigeria, after 20-24 month of

451 massive used of dieldrin, homozygote resistant mosquitoes (RR) was the only ones remaining
452 in the *An. gambiae* population with only few heterozygotes (RS). But, after six months in the
453 absence of dieldrin, the homozygote susceptible genotype took over.
454 Similar results were reported for *An. culicifacies* in India (Bhatia and Deobhankar 1963).
455 However, the same allele at the *rdl* locus has been reported to be maintained in field
456 populations in Sri Lanka despite the withdrawal of cyclodiene insecticides for mosquito
457 control for more than 30 years (Roush and McKenzie 1987). Altogether these variations
458 indicate that knowledge of the reversal rate of an insecticide is crucial for implementing any
459 resistance management strategy in the field based on rotation of insecticides.
460
461 **Conclusion**
462 This study has investigated the fitness cost of P450-based metabolic resistance to
463 pyrethroids in a major malaria vector revealing significant fitness cost for fecundity, fertility
464 and the larval development of resistant mosquitoes. This fitness cost was further supported by
465 the observation of a return to susceptibility in the absence of insecticide over 10 generations
466 (around 1 year) showing that if suitable resistance management strategies such as rotation was
467 implemented, P450-based resistance could be managed. This should encourage future
468 strategies using non pyrethroid-based LLINs to reduce the selection pressure and allow such
469 rotation to slow the spread of pyrethroid resistance.
470
471
472 **Author Contributions**: C.S.W. conceived and designed the study; Ma.T carried out the
473 sample collection; Ma.T, D.D., W.T. reared and maintained the strain in the insectary;
474 Ma.T. Mi.T, L.M.J.M. and M.J.W performed the molecular analyses; Ma.T, J.M.R and

C.S.W. analyzed the data; Ma.T and C.S.W. wrote the manuscript with contributions from F.N.. All authors read and approved the manuscript.

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**Data Archiving:** Underlying data is available here

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642

**Figure legends**

**Figure 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; (*) 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/FUMOZ-R represents a line obtained from the crossing between female FANG and male FUMOZ-R whereas FUMOZ-R/FANG represents a line obtained from the crossing between female FUMOZ-R and male FANG; Perm is permethrin and Delt is deltamethrin.

**Figure 2. Fecundity and fertility of females with different genotypes of the *CYP6P9a* gene**: (A) and (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.

**Figure 3. Distribution of the *CYP6P9a* genotypes at different time-points of the development of immature stages**. (**A**) Stacked bar plot of the variation in genotypes 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 (day1) to formation of the pupae; (**D**) the proportion of pupae obtained in day 9, day 11 and day 13 of development. Colored bars and lines indicate respectively *CYP6P9a*-RR, *CYP6P9a*-RS and *CYP6P9a*-SS genotypes.

**Figure 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; (*) indicates statistically significant differential expression of the gene in comparison with susceptible strain; *NS*: not significant.

**Figure 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 generation in the insecticides free-environment. F represents each generation; dotted line indicates a frequency of 50% for the resistant and susceptible alleles.

**Table 1.** Assessment of the association between *CYP6P9a* genotypes and the ability of females to lay eggs. SS: homozygote susceptible; RR: homozygote resistant; RS: heterozygote; (*) significant difference $p < 0.05$.

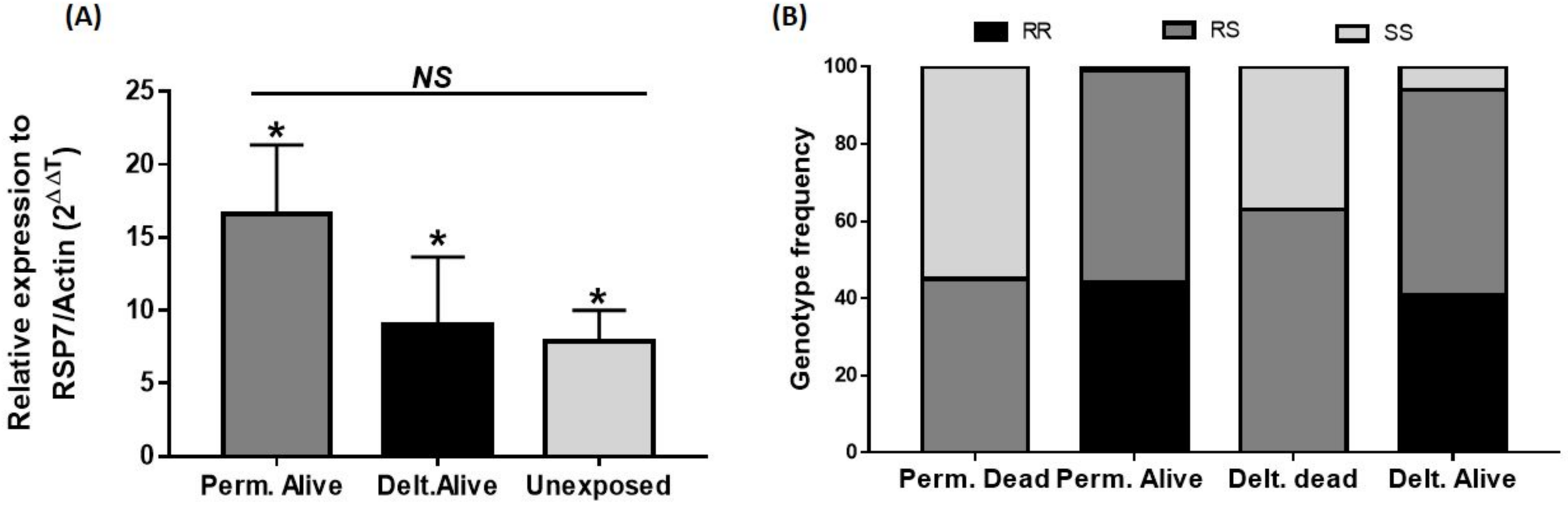
| Combination of genotypes at the *CYP6P9a* locus | Level of association | |
|---|---|---|
| | **Odds ratio** | ***p*-value** |
| **SS vs. RR** | 2.0 (0.7–5.7) | 0.15 |
| **SS vs. RS** | 2.04 (1.1–3.8) | 0.01 * |
| **RS vs. RR** | 1 (0.4–2.6) | 0.57 |
| **S vs. R** | 1.4 (0.8 –2.6) | 0.1 |

**Table 2.** Association between *CYP6P9a* genotypes/alleles and pupae formation; (*), significant difference.

| Combination of genotypes at the *CYP6P9a* locus | $Day_{9}$ x $Day_{11}$ | | $Day_{9}$ x $Day_{13}$ | | $Day_{11}$ x $Day_{13}$ | |
|---|---|---|---|---|---|---|
| | **Odds Ratio** | ***p-value*** | **Odds Ratio** | ***p-value*** | **Odds Ratio** | ***p-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* |

704

705


(A)
NS
*
*
*
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Relative expression to RSP7/Actin ($2^{\Delta\Delta T}$)
Perm. Alive
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Unexposed
(B)
RR
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SS
100
80
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Genotype frequency
Perm. Dead
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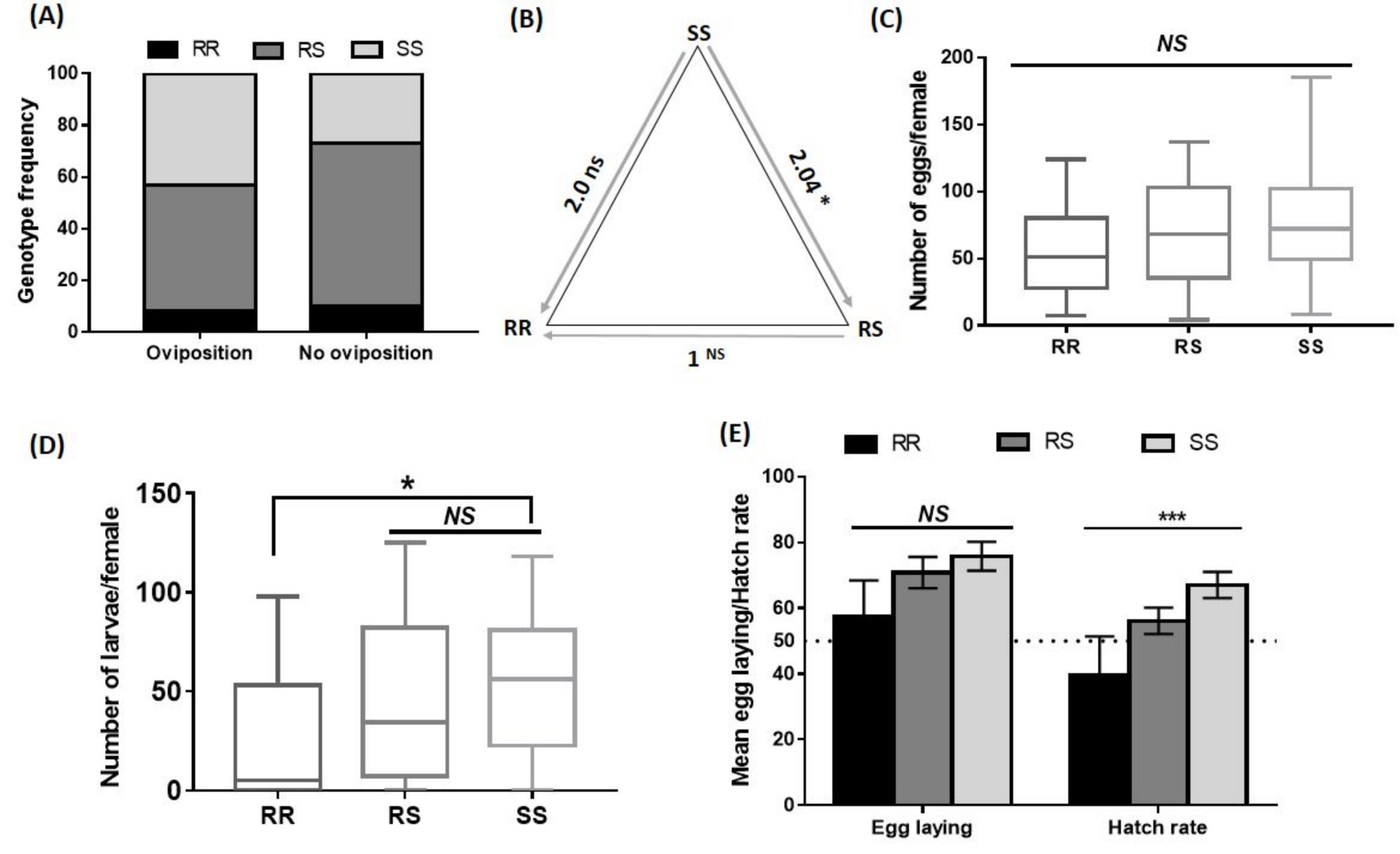
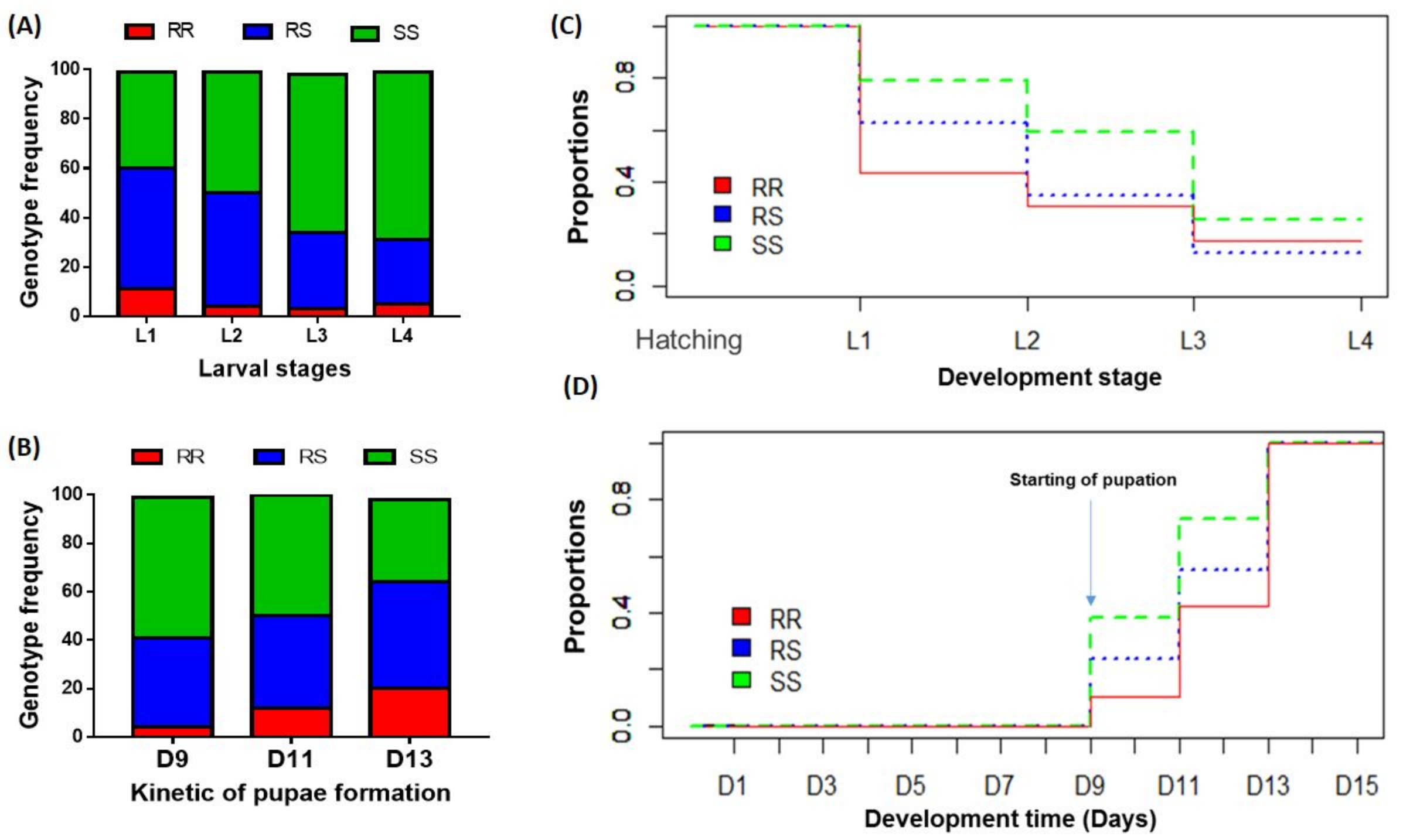
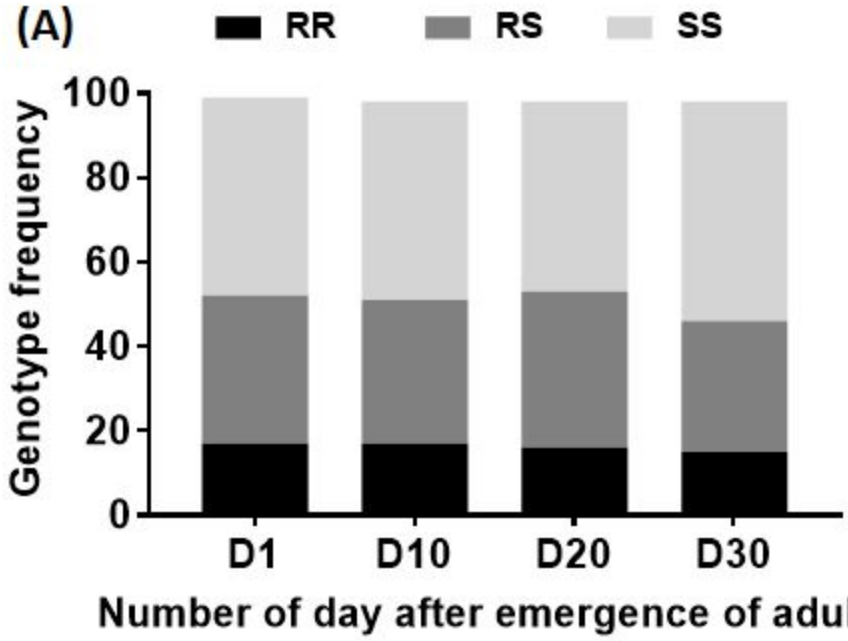
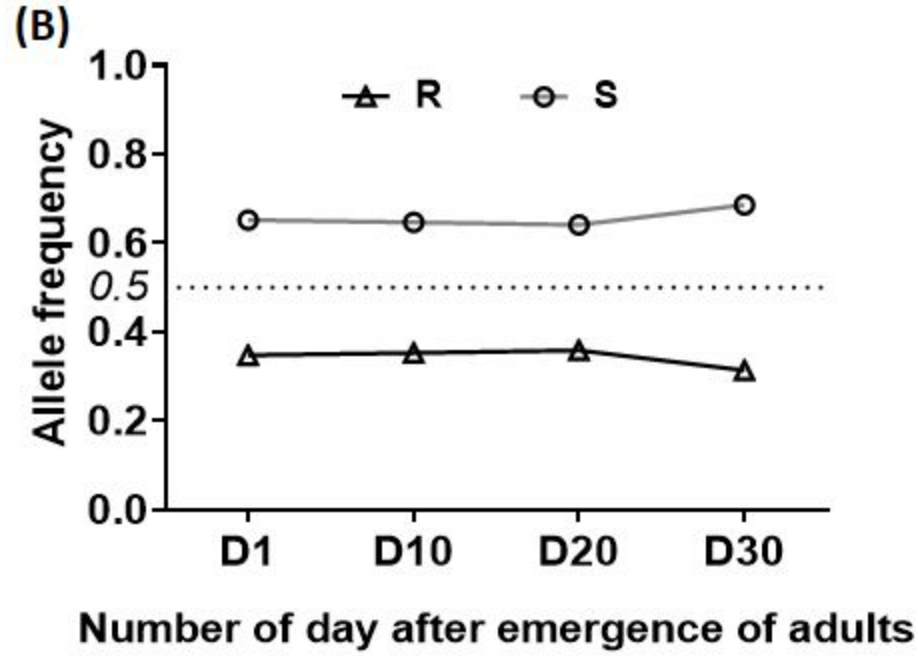
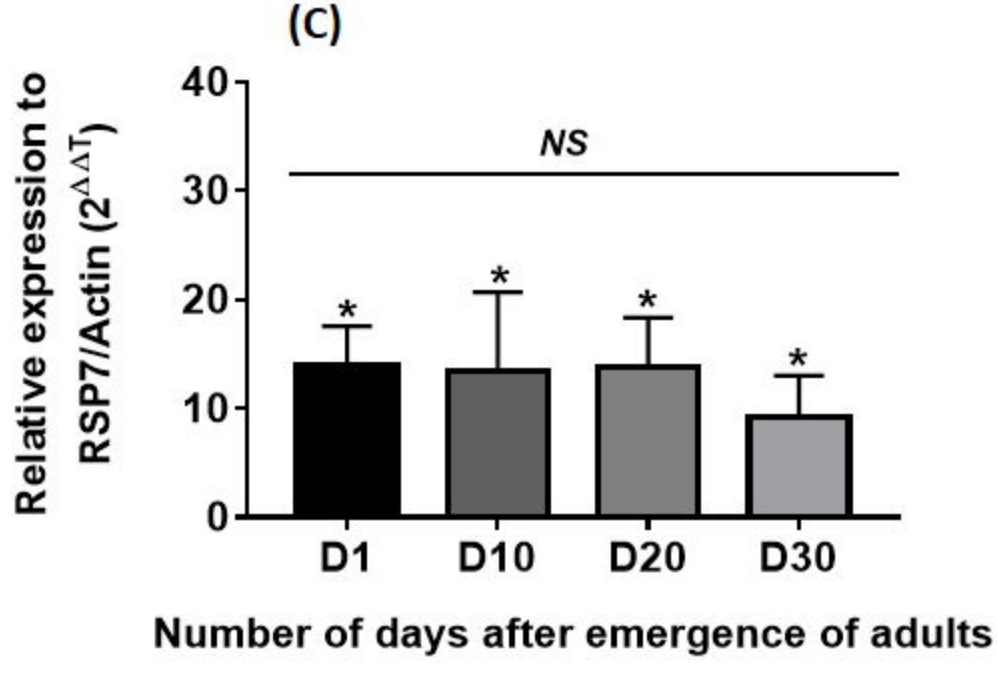
Delt. dead
Delt. Alive

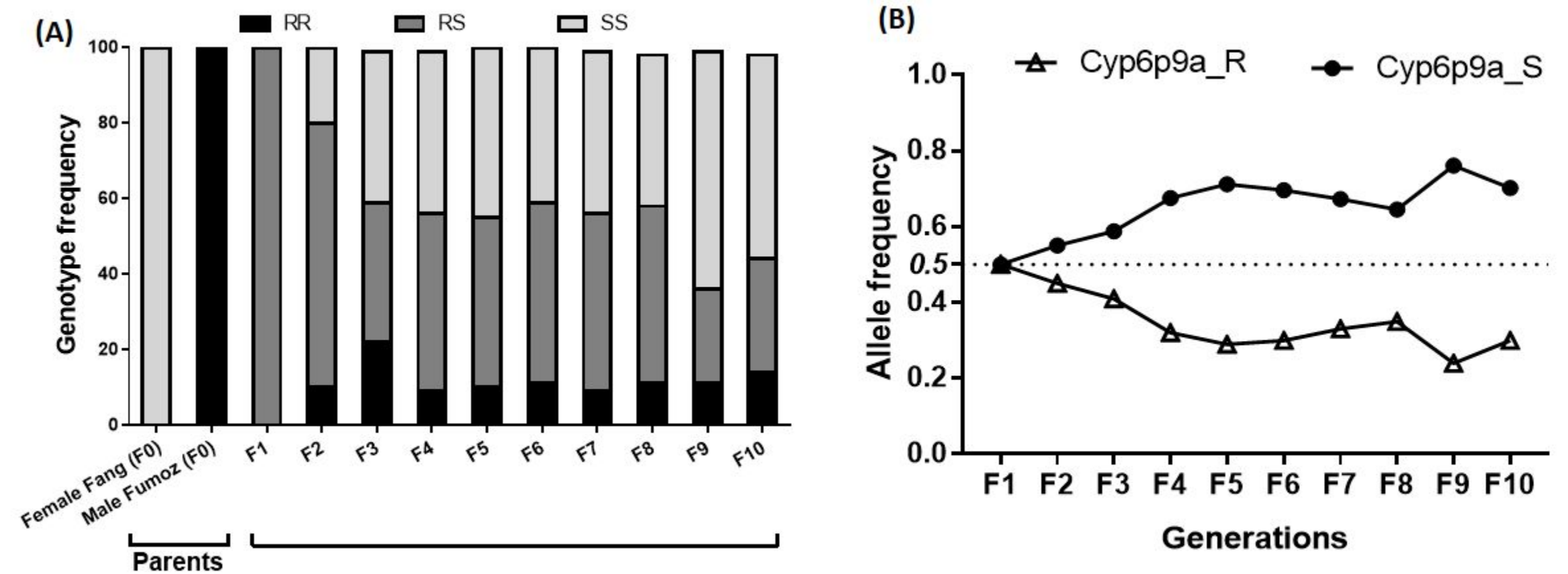

(A)
RR
RS
SS
Genotype frequency
100
80
60
40
20
0
Oviposition
No oviposition
(B)
SS
2.0 ns
2.04 *
RR
RS
1 NS
(C)
NS
Number of eggs/female
200
150
100
50
0
RR
RS
SS
(D)
*
NS
Number of larvae/female
150
100
50
0
RR
RS
SS
(E)
RR
RS
SS
Mean egg laying/Hatch rate
100
80
60
50
40
20
0
NS
***
Egg laying
Hatch rate


(A)
RR
RS
SS
Genotype frequency
100
80
60
40
20
0
L1
L2
L3
L4
Larval stages
(B)
RR
RS
SS
Genotype frequency
100
80
60
40
20
0
D9
D11
D13
Kinetic of pupae formation
(C)
Proportions
0.8
0.4
0.0
RR
RS
SS
Hatching
L1
L2
L3
L4
Development stage
(D)
Starting of pupation
Proportions
0.8
0.4
0.0
RR
RS
SS
D1
D3
D5
D7
D9
D11
D13
D15
Development time (Days)


(A)
RR
RS
SS
Genotype frequency
100
80
60
40
20
0
D1
D10
D20
D30
Number of day after emergence of adults


(B)
R
S
Allele frequency
1.0
0.8
0.6
0.5
0.4
0.2
0.0
D1
D10
D20
D30
Number of day after emergence of adults


(C)
Relative expression to RSP7/Actin ($2^{\Delta\Delta T}$)
NS
40
30
20
10
0
*
*
*
*
D1
D10
D20
D30
Number of days after emergence of adults


(A)
RR
RS
SS
Genotype frequency
100
80
60
40
20
0
Female Fang (F0)
Male Fumoz (F0)
F1
F2
F3
F4
F5
F6
F7
F8
F9
F10
Parents
(B)
Cyp6p9a_R
Cyp6p9a_S
Allele frequency
1.0
0.8
0.6
0.5
0.4
0.2
0.0
F1
F2
F3
F4
F5
F6
F7
F8
F9
F10
Generations