Insecticide Resistance Exerts Significant Fitness Costs in Immature Stages of Anopheles Gambiae in Western Kenya.

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Research

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Abstract

Background: Despite increasing documentation of insecticide resistance in malaria vectors against public health insecticides in sub-Saharan Africa, there is a paucity of information on the potential fitness costs of pyrethroid resistance in malaria vectors which is important in improving the current resistant management strategies. This study aimed to assess the fitness cost effects of insecticide resistance on the development and survival of immature *Anopheles gambiae* from western Kenya.

Method: Two-hour old first instar larvae (L1) were introduced and raised in basins containing soil and rainwater in a semi-field setup. Each day the number of surviving individuals per larval stage was counted and their stage of development were recorded until they emerged as adults. The larval life history trait parameters measured include mean larval development time, daily survival and pupal emergence. Pyrethroid selected resistant colony of *An. gambiae s.s* and unselected colony originating from the same site and with same genetic background were used. Kisumu laboratory susceptible colony was used as a reference.

Results: The selected resistant colony had a significantly longer larval development time through the developmental stages than the unselected susceptible colony. The selected colony took an average of 2 days longer to develop from first instar (L1) to fourth instar (L4) (8.8 ± 0.2 days) compared to unselected colony (6.6 ± 0.2 days). The development time from first instar to pupa formation was significantly longer by 3 days in the selected colony (10.28 ± 0.3 days) than in unselected colony (7 ± 0.2 days). The time from egg hatching to adult emergence was significantly longer for the selected colony (12.1 ± 0.3 days) than the unselected colony (9.2 ± 0.2 days). The pupation rate (80%; 95% [CI: 77.5-83.6] vs 83.5%; 95% [CI: 80.6-86.3]) and adult emergence rate (86.3% vs 92.5%) did not differ between the selected and unselected colonies respectively. The sex ratio of the females to males for the selected (1:1.21) and unselected colonies (1:1.07) was significantly different.

Conclusion: The study showed that pyrethroid resistance in *An. gambiae* had a fitness cost on their pre-imaginal development time and survival. Insecticide resistance delayed the development and reduced the survivorship of *An. gambiae* larvae. The study findings are important in understanding the fitness cost of insecticide resistance vectors that could contribute to shaping resistant management strategies.

Background

The development and spread of insecticide resistance threaten the control of vectors of infectious diseases in sub-Saharan Africa (1). The continued use of insecticides for public health interventions and agricultural purposes seems to have generated high selective pressure on mosquito populations leading to the development of insecticide resistance in mosquito vectors (2–4). Resistance to insecticides in malaria vectors has mainly been linked to the overexpression of detoxifying enzymes or enzyme structural changes that increase metabolic activity and target-site modification (5, 6). This ability to resist insecticides through different mechanisms may present a fitness cost to resistant genotypes with

negative effects in their development, reproductive aspects and vector competence which could affect the vectorial capacity of the malaria vectors (7).

Environmental selection pressure may select for certain phenotypes that will adapt to the new environment. It is hypothesized that phenotypic changes in an organism may have deleterious effects when the organism returns to its old environment (8). For instance, resistant mosquito genotypes are believed to have an adaptive advantage in the insecticide environment resulting in increased resistance levels and this tends to decrease in the absence of insecticides suggesting the existence of a fitness cost (9). The development and maintenance of resistant mechanisms in mosquitoes are thought to divert energy and resources associated with the primary physiological process, such as fecundity and longevity of individuals leading to a biological cost (8, 10). Overexpression of metabolic enzymes and genes in resistant mosquitoes are thought to reallocate primary energetic resources from other life-history traits e.g. egg production and larval development to maintain secondary metabolic pathways involved in defence resulting in a fitness cost (11). Changes in the insecticide target site may result in a fitness cost if the molecular alteration or the expressed genes are essential for the viability of the insect impairing the resistant individuals' development and reproductive fitness (9)

Fitness costs associated with resistance have been reported to affect primary biological characteristics in resistant *Culex* and *Aedes* mosquitoes (9, 12–17). Studies by Alout et al(18) have also documented the fitness cost of insecticide resistance alleles on the vector competence of resistant phenotypes (19). Currently, little is known about the effects of major insecticide resistance mechanisms on the life-history parameters of *An. gambiae* the major malaria vector in Africa. This presents a gap that needs to be filled for better designing of malaria control strategies as insecticide resistance is increasingly reported, threatening the already achieved progress in malaria reduction in Africa. This study assessed the fitness cost effects of insecticide resistance on the development and survival of immature An. gambiae from western Kenya.

Materials And Methods

Mosquito population used in the study

The colonies of *An. gambiae s.s* used in this study consisted of a pyrethroid-resistant selected colony (hereafter R colony) originating from Bungoma in western Kenya and an unselected colony (hereafter S colony) from the same site (Machani et al, 2020, in press). The R colony was selected using 0.05% deltamethrin at every generation. The S colony was not selected at any generation but a subset of this colony was checked for resistance after every other generation. The sixth generation of the R colony and the thirteenth generation of the S colony were used for this study. The 6th generation of the R colony used had an average mortality rate of 20% whilst the 13th generation of the S colony had an average mortality rate of 97.3% when exposed to 0.05% deltamethrin (supplementary Fig.1). The S colony had almost lost resistance after 9 generations without insecticide selection pressure (Mortality: 92%). The two knockdown resistance mutations (kdr) L1014S and L1014F were at high frequency (0.77 and 0.23 respectively) in the

R colony compared to the S colony (L1014S-0.98; L1014F-0) (Machani et al 2020, in press). Monooxygenase played a major role in resistance in the selected colony used, with an 11% increase in the enzyme activity compared to the unselected colony that exhibited a 42% reduction in the enzyme activity in relation to the parent population. The *An. gambiae* Kisumu reference laboratory strain which has been colonized since 1954 and is free of any detectable insecticide resistance mechanism was used as a control susceptible strain in all bioassays.

The mosquito colonies were reared in the insectary at KEMRI/CGHR under standard conditions (25 ± 2 °C; 80% ± 4% relative humidity with a 12 h: 12 h light/dark cycle). Larvae were fed on tetramin baby fish food and brewer's yeast daily and adults maintained in a 10% sugar solution.

Life table experiments

Three parameters were evaluated to examine the fitness cost: mean larval development time (L1-Pupal), pupal emergence) and daily survival. The parameters were measured under semi-field conditions and focused on the difference between mosquitoes raised in the presence (selected colony) and the absence of insecticide selection pressure (unselected colony). The Kisumu susceptible laboratory strain was used as a control.

Experimental design

A total of 27 semi-natural habitats (9 replicates per colony) were created using plastic washbasins (35 cm in diameter and 15 cm deep) at KEMRI/CGHR compound in Kisumu according to the method described by Afrane *et al* (20). Two kilograms of soil from breeding sites and five litres of rainwater were added to each washbasin. Two holes (3 cm in diameter) were created near the top edge of each washbasin to maintain a constant water level when it rained. The holes were covered with a screen (mesh size _ 200 micrometres) to prevent larvae from being washed away (21, 22). Thirty (30) 2-hour old larvae from the three colonies were placed separately in different basins. Each washbasin was covered with a nylon netting to prevent predators and wild mosquitoes from ovipositing eggs in the washbasin. The surviving larvae in each washbasin were checked and counted daily and their numbers were recorded. The stage of development of individual larvae was also recorded to measure the development time per each larval instar. Pupae were picked, recorded and transferred to pupal cups, which were then placed in cages for adult emergence. Pupae were monitored daily and the number and sex of emerging adults recorded. All larvae from the three colonies were reared through adults in semi-field conditions. The mean length of time from the first instar to adult emergence for each sex, as well as the ratio of male to female emergences was recorded for each colony. The experiment was repeated four times.

Data analysis

Mean larval development time was defined as the average time of the first instar larvae to develop into adults. Mean pupation time was calculated as the average time taken for the first instar larva to pupate. The male and female development time was recorded differently because they take different times to

emerge. The pupation rate was calculated as the percentage of the first instar larvae that emerged to pupae. The emergence rate was calculated as the percentage of the pupae that emerged to adults. Analysis of variance (ANOVA) was conducted to determine the effects of insecticide resistance on the pupation time, larval development time, pupation rate and emergence rate of the R colony, S colony and the Kisumu reference *An. gambiaes.s*. Tukey HSD post hoc tests were used to determine the statistical significance of the difference in larval development time, pupation rate and emergence rate among the selected, unselected and the Kisumu reference colonies. Kaplan-mier survival test was used in the testing for differences in larval survivorship among the selected, unselected and the Kisumu reference mosquitoes. The level of significance was set at 0.05 for all tests.

Results

Effect of insecticide resistance on larval development

The mean development time from first instar (L1) to second instar (L2) in the R colony was 4.2 ± 0.2, while the S colony was 3.4 ± 0.1 and 3.4 ± 0.1 for the Kisumu strain ($F_{2,63}$=44.43, P < 0.0001; Table 1). The average length of larval development time (L1-L2) for the resistant colony was 0.8 days longer compared to the unselected colony. The time for R colony to develop from first instar (L1) to third instar (L3) was 6.9 ± 0.2 days while the S colony was 4.9 ± 0.2 and 4.8 ± 0.2 days for the Kisumu colony. The development time (L1-L3) for R colony was 2 days longer compared to the other colonies ($F_{2,63}$=44.61, P < 0.0001). The mean pre-imaginal development time from first instar (L_1) to fourth instar (L_4) of the R colony was 8.8 ± 0.2, while the S colony was 6.6 ± 0.2 and 6.3 ± 0.2 for Kisumu laboratory susceptible mosquitoes. The R colony took a significantly longer period (2 days) to develop from L_1-L_4 with respect to the S colony and Kisumu strain ($F_{2,63}$=47.06, P < 0.0001).

Table 1
Comparison of larval instar development time among the R, S and Kisumu colonies

Population	Larval instar development time (days)		
	L2[i]	L3[ii]	L4[iii]
	Mean ± SE	Mean ± SE	Mean ± SE
R colony	4.9 ± 0.2[b]	6.9 ± 0.2[b]	8.8 ± 0.2[b]
S colony	3.4 ± 0.1[a]	4.9 ± 0.2[a]	6.6 ± 0.2[a]
Kisumu strain	3.4 ± 0.1[a]	4.8 ± 0.2[a]	6.3 ± 0.2[a]

* Values indicate mean and the standard error. The same superscript letters in each row indicate no significant difference (p > 0.05, ANOVA, followed by Tukey (HSD) test. [i] Duration of L1 to develop to the second instar larvae (L2). [ii] Duration of L1 to develop into the third instar larvae (L3). [iii] Duration of L1 to develop into the fourth instar larvae (L4).

Pupation and emergence times between the selected and unselected colonies

The R colony reached pupal stage 10–11 days after hatching as L1, whilst the S colony took 6–7 days. Development time from L1 to pupal stage was significantly longer in R colony than in the S colony (mean: 10.28 ± 0.3 vs 7.5 ± 0.2; $F_{2,63}$=39.45, P < 0.0001, Table 2). The Kisumu strain took 7.9 ± 0.2 days to pupate.

Table 2
Comparison of larval - life trait parameters of the Kisumu, selected and the unselected *An. gambiae s.s* colonies

Population	Pupation time (days)[1]	Pupation rate (%)[2]	Mean development time of Male (days)[3]	Mean development time of females (days)[4]	Emergence rate (%)[5]
R colony	10.28 ± 0.3^{b}	80 ± 0.0^{a}	11.9 ± 0.30^{b}	12.1 ± 0.3^{b}	86.3 ± 0.0^{a}
S colony	7.5 ± 0.2^{a}	83.5 ± 0.0^{a}	9.2 ± 0.2^{a}	9.6 ± 0.2^{a}	92.8 ± 0.0^{a}
Kisumu strain	7.9 ± 0.2^{a}	84.5 ± 0.0^{a}	9.4 ± 0.2^{a}	9.8 ± 0.2^{a}	85.7 ± 0.0^{a}

* Values indicate mean and the standard error. The same superscript letters in each row indicate no significant difference (p > 0.05, ANOVA, followed by Tukey (HSD) test. [1] Duration of L1 larvae developing to pupae.[2]Percent of larvae developing to pupae. [3]Duration of L1 larvae to develop to males [4]Duration of L1 to develop into females. [4]. [5] Percent of pupae developing to adults.

The pupation rate in the R colony was 80% (95% CI: 77.5–83.6), while it was 83.5% (95% CI: 80.6–86.3) for the S colony. Although the R colony took a longer time to develop, there was no significant difference in the pupation rate between R and S colonies ($F_{2,63}$=0.084, P > 0.05).

The proportion of pupae emerging to adults was high in S colony 92.5% compared to the R colony 86.3%. However, this was not statistically significant ($F_{2,63=}$7.18, P > 0.05). The emergence rate for the Kisumu strain was 85.7% (Table 2). The emergence time for males and females in R colony was 11.9 ± 0.3 and 12.1 ± 0.3 respectively while the S colony was 9.2 ± 0.2 and 9.8 ± 0.2 days respectively. The male emergence time for the Kisumu strain was 9.4 days and 9.8 days for females. There was a significant difference between the emergence time for males and females in the R colony compared to S colony (Males; $F_{2,63}$=38.4, P < 0.05; Females, $F_{2,63}$=35.81, P < 0.05, Table 2).

The proportion of males emerged from R and S colonies were higher (R colony: 54.8% (95% CI: 50.4–59.3); S colony: 51.7% (95% CI: 47.2–56.2) compared to the emerged females (R colony: 45.2% (95% CI: 40.7–49.6); S colony: 45.5% (95% CI: 41.2–50) (Table 3). The sex ratio of females to males was significantly different for R colony 1: 1.21 (t = 2.5248, df = 42, P < 0.0154) and S colony 1: 1.07 (t = 2.2525,

df = 42, P < 0.029). Although the proportions of males to females was high in the Kisumu strain (51.7 vs 48.3%), this was not statistically significant (t = 0.854, df = 42, P > 0.05).

Table 3
The average number of male and female adults emerged from the three colonies.

Colony	Sample size	Female	Male	Sex ratio
		Mean (%) ± SD[i]	Mean (%) ± SD[ii]	Female: Male[iii]
R colony	485	45.2 ± 0.6	54.8 ± 0.6	1:1.2[a]
S colony	512	45.5 ± 0.5	54.5 ± 0.7	1:1.19[a]
Kisumu strain	472	48.3 ± 0.6	51.7 ± 0.6	1 :1.07[b]

* Values indicate mean and the standard error. The same superscript letters in the last row indicate no significant difference. [i] Proportion of females that emerged. [ii] Proportion of males that emerged. [iii] Sex ratio of females to males.

Survivorship Among The Selected And Unselected

The R colony showed a longer survival time of 15 days, with a median survival length of 8 days compared to the S colony that survived for 12 days with a median survival length of 6 days (Fig. 1). The Kisumu strain exhibited a very similar trend as the S colony. When comparing the survival curves using Wilcoxon proportional hazard ratio test, there was no significant difference in larval survivorship between R and S colonies (P = 0.43). The larval mortality rate was high in the R colony 20% (95% CI: 16.4–22.5), while in the S colony was 16% (95% CI: 13.5–19.2) and Kisumu strain was 17% (95% CI: 13.7–19.3). Although the mortality rate was high in the R colony, no significant difference was found between the colonies ($F_{2,39}$=0.141, P > 0.05).

Discussion

Under an evolutionary perspective, it is hypothesised that genetic changes arising as a result of insecticides' selective pressure can present a fitness cost to resistant insects bearing negative effects on their biological traits (23). The study assessed larval development time and survivorship of *An. gambiae* colonies, exhibiting different insecticide resistance status. The results of this study demonstrate the existence of fitness cost n *An. gambiae s.s* immature stages associated with pyrethroid resistance. Overall larval development time and survival was compromised in the pyrethroid-selected colony compared to the unselected colony originating from the same background. The development time of the unselected colony was remarkably similar to that of the susceptible Kisumu reference strain.

The study observed prolonged development time from one larval instar to the other in the selected resistant colony when compared with the unselected colony whose development time was similar to the

Kisumu colony. Majority of individuals from the unselected colony and the Kisumu strain reached the pupal stage in 6–7 days after the hatching of the first instar whereas the selected colony took additional 3–4 days before pupation. These findings present adaptive disadvantage on the resistant individuals as the amount of time spent in the natural breeding habitats in the field may impact their survival rates due to exposure to natural predations. They are also likely to suffer temporary or permanent loss of habitats before emerging to adults, which may in turn have a direct consequence on the vectorial capacity (7). Similarly, studies on pyrethroid-resistant *An. funestus*, *Culex quinquefasciatus, Aedes aegypti* and *Aedes albopictus* have observed longer phase of larval development unlike their susceptible counterparts (12, 14–16, 24).

Larval survivorship of the pyrethroid-resistant colony was low characterized by low pupation rates, high pupae mortality and decreased adult emergence compared to the unselected and Kisumu colonies. These could be possibly due to the accumulation of harmful effects of genes related to insecticide detoxifying enzymes or molecular alterations on the target (kdr mutations). The success in survivorship of the unselected or susceptible colony could be attributed to the loss of resistance in them that could enable them to focus most of their energy to growth enhancement metabolic processes. The low larval survivorship in the selected colony may present low vector population densities disabling effective malaria transmission by resistant mosquitoes. Similar studies have reported the negative effects associated with insecticide resistance on the biological characteristics of pyrethroid-resistant *Aedes albopictus* and *Culex pipiens* compared to their susceptible counterparts (17, 25)

It is important to highlight that monooxygenase enzyme was majorly implicated in the pyrethroid resistance of the selected colony even though kdr mutations were observed at high frequencies (Machani et al 2020, in press). It is likely that the overproduction of monooxygenase would have committed resources important for primary biological functions such as development to maintaining secondary functions i.e. insecticide detoxification (23). For instance, some studies have linked the staggered larval development time of resistant individuals with spending more time in the accumulation of nutrients to achieve the development threshold that triggers growth to the next stage as most of the resources are used to maintain resistance (26). The findings of this study are similar to reports on *An. funestus* from West Africa harboring 119F-GSTe2 resistant alleles which exhibited delayed larval development compared to the population without the resistant alleles (27). The *kdr* mutation has been associated with a delay in the larval development of *A. aegypti*, (9, 28). The observed negative effects associated with insecticide resistance may affect the spread of insecticide resistance genes in a population, as the resistant individuals are likely to take a longer time to develop and emerge as adults, unlike the susceptible ones. Based on this, resistance management tactics may rely on this reduced fitness disadvantage to design integrated vector control management strategies with an aim of limiting the spread of insecticide resistance and maintaining the effectiveness of the existing vector control tools.

Conclusion

This study revealed that there was a fitness cost associated with pyrethroid resistance in *An. gambiae*. Pyrethroid resistance resulted in fitness disadvantages as exhibited by the selected colony that recorded slow larval development time and reduced survivorship. These negative fitness aspects were linked to the increased frequencies of kdr mutations and high monooxygenase and esterase enzyme detoxification activities in the selected *An. gambiae.* These findings could be useful in developing better insecticide management strategies.

Declarations

Authors' contribution

JO, MM, EO and YAA conceived and designed the experiments, JO and MM participated in data collection, data analysis and drafted the manuscript. EO, CW, EO, SM, AK, and YAA supervised data collection and contributed to manuscript writing. All authors have read and approved the final manuscript.

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Consent for publication

The permission to publish this study was granted by the director of Kenya Medical Research Institute.

Competing interest

The authors declare that they have no competing interests.

Availability of data and materials

The dataset supporting the conclusions of this article is included within the article

Ethics approval and consent to participate

The study was approved by the Ethical Review Board of the Kenya Medical Research Institute (KEMRI) under the scientific steering committee (SSC 3434).

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Figures

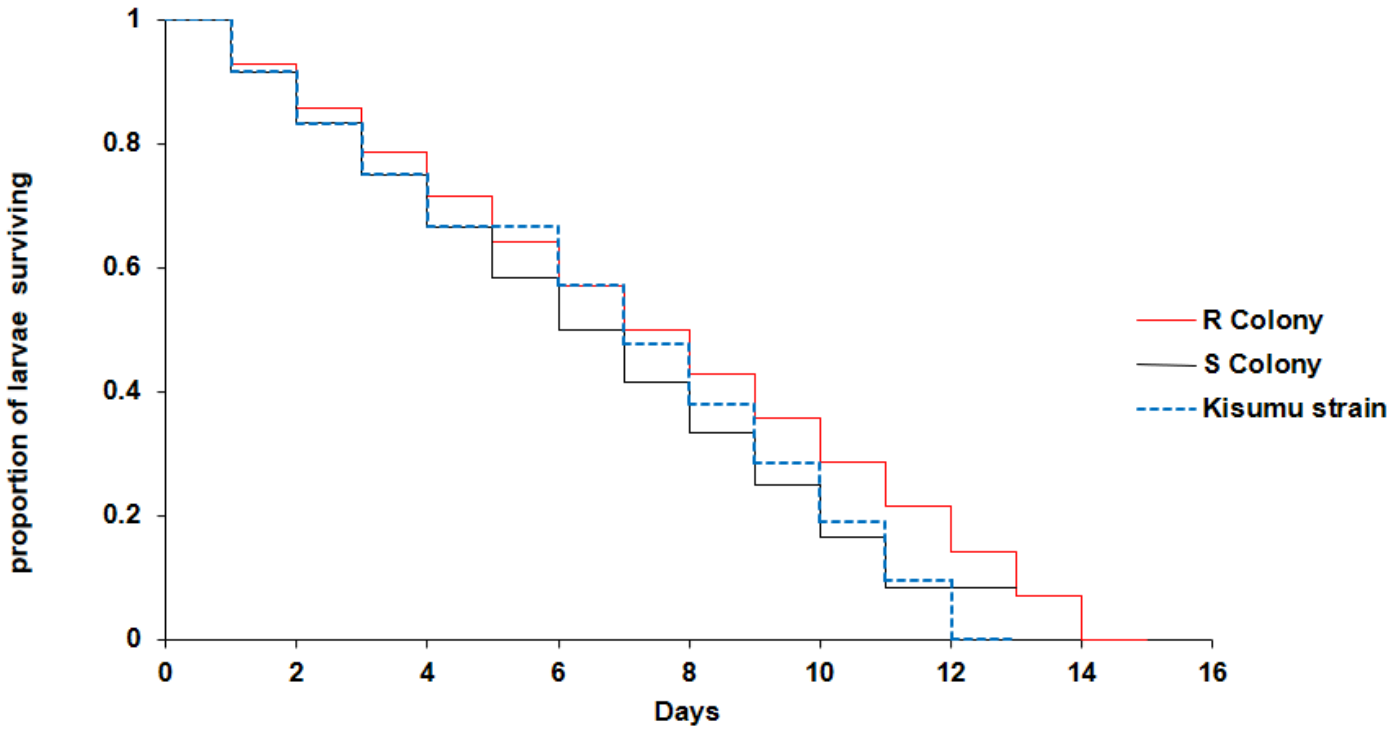

Figure 1

Larval survival probability of pyrethroid-selected resistant and unselected colonies of Anopheles gambiae s.s

Supplementary Files

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