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Is *Anopheles gambiae* (*sensu stricto*), the principal malaria vector in Africa prone to resistance development against new insecticides? Outcomes from laboratory exposure of *An. gambiae* (*s.s.*) to sub-lethal concentrations of chlorfenapyr and clothianidin

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ABSTRACT

Indiscriminate use of pesticides in the public health and agriculture sectors has contributed to the development of resistance in malaria vectors following exposure to sub-lethal concentrations. To preserve the efficacy of vector control tools and prevent resistance from spreading, early resistance detection is urgently needed to inform management strategies. The introduction of new insecticides for controlling malaria vectors such as clothianidin and chlorfenapyr requires research to identify early markers of resistance which could be used in routine surveillance. This study investigated phenotypic resistance of *Anopheles gambiae* (*sensu stricto*) Muleba-Kis strain using both WHO bottle and tube assays following chlorfenapyr, clothianidin, and alpha-cypermethrin selection against larvae and adults under laboratory conditions. High mortality rates were recorded for both chlorfenapyr-selected mosquitoes that were consistently maintained for 10 generations (24-h mortality of 92–100% and 72-h mortality of 98–100% for selected larvae; and 24-h mortality of 95–100% and 72-h mortality of 98–100% for selected adults). Selection with clothianidin at larval and adult stages showed a wide range of mortality (18–91%) compared to unselected progeny where mortality was approximately 99%. On the contrary, mosquitoes selected with alpha-cypermethrin from the adult selection maintained low mortality (28% at Generation 2 and 23% at Generation 4) against discrimination concentration compared to unselected progeny where average mortality was 51%. The observed resistance in the clothianidin-selected mosquitoes needs further investigation to determine the underlying resistance mechanism against this insecticide class. Additionally, further investigation is recommended to develop molecular markers for observed clothianidin phenotypic resistance.

1. Introduction

Control of malaria and arbovirus vectors for the last two decades has been reliant on the use of bednets, indoor sprays, and larvicides based mainly on pyrethroids. The use of insecticide-treated nets (ITNs) and indoor residual spraying (IRS) for example, has accounted for more than 70% of the malaria reduction between 2000 and 2015 (Cibulskis et al., 2016). However, these gains are highly undermined by the spread of pyrethroid resistance in malaria vectors which has been reported in all malaria-endemic countries (Ranson et al., 2011; Hemingway, 2018). Similarly, pyrethroid resistance in arbovirus vectors, especially *Aedes aegypti* and *Aedes albopictus*, has been reported widely (Ayorinde et al., 2015; Braack et al., 2018; Amelia-Yap et al., 2018; Demok et al., 2019). Development of resistance to pyrethroids is mainly mediated by metabolic resistance and target-site mutations that may cause cross-resistance with other insecticide classes (Brengues et al., 2003; Moyes et al., 2021).

Insecticide resistance is a genetic change within an organism in response to selection by the insecticide, subsequently resulting in failure of vector control (Guedes, 2016). Insecticide resistance occurs as a result

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of selection effect from insecticide exposure through differential mortality, or differential survival and reproduction among individuals. The resistance development is attributed to application of insecticides in the public health and agricultural sectors which is associated with irregular or sub-standard deployment and decaying concentration (Hardin et al., 1995; Macfadyen et al., 2014). This shortcoming is not restricted to conventional (synthetic) insecticides but occurs also in bioinsecticides, reduced-risk insecticides, and insecticidal proteins (Guedes, 2016). Although the insecticide application could initially cause quick mortality of a targeted pest species, its residue degrades over time reducing the original (lethal) deposit to a (sub-lethal) deposit capable of exhibiting biological effects on targeted or non-target pest species (Gressel, 2010).

While the use of lethal insecticide concentrations eliminates susceptible individuals, sub-lethal exposure favors the survival and reproduction of the resistant individuals, resulting in insecticide resistance. The mechanism by which sub-lethal insecticide exposure causes insecticide resistance development is categorized into three areas. First, it may delay selection for major single-gene resistance while favoring polygenic resistance (Gressel, 2010) resulting from the accumulation of low-level resistance genes and mechanisms leading to increased resistance (Gressel, 2010; Nansen et al., 2016). Also, sub-lethal exposure facilitates increased mutation rates (Gressel, 2010; Torres-Barceló et al., 2013; Ram and Hadany, 2014).

Another way in which sub-lethal insecticide exposure may impact insecticide resistance is through induction of detoxification enzymes. Detoxification enzymes when upregulated and overexpressed within resistant pest populations have been responsible for resistance against insecticides such as pyrethroids and neonicotinoids (Nardini et al., 2012). Induction through sub-lethal exposures, by either susceptible or resistant insects stimulates them against further exposure to the same or different insecticides allowing enhanced insecticide tolerance (Poupardin et al., 2008; Bass et al., 2015; Rix et al., 2015). There are already reports on the selection for resistance as a result of the priming effect in mosquitoes with a history of sub-lethal exposure to urban pollutants and agriculture pesticides (Poupardin et al., 2012; Nkya et al., 2013, 2014). Depending on the dosage of exposure to sub-lethal insecticide concentration, organisms can suffer expected detrimental effects, or beneficial effects to either the exposed organism or its progeny, depending on the fitness cost involved (Cutler and Rix, 2015; Douglas, 2015). Penetration of sub-lethal insecticide into an organism will likely affect its physiology causing insecticide resistance (Guedes, 2016).

Evolution and spread of resistance have undermined the efficacy and sustainability of pyrethroid-, carbamate-, organophosphate- and organochlorine-based tools (Brogdon et al., 1999; Ibrahim et al., 2016; Mugenzi et al., 2023) creating a shortage of available insecticides for vector control. To fill this gap, the Global Plan for Insecticide Resistance Management listed the development of new insecticides with different modes of action as a requirement to manage insecticide resistance in malaria vectors *via* rotations (WHO, 2012). Consequently, new insecticides with different modes of action or combinations with synergists are being developed to complement current tools for the control of pyrethroid-resistant mosquitoes (Oxborough et al., 2015; Mbewe et al., 2023; Snetselaar et al., 2023). The limited number of insecticide classes approved for ITN treatments means there are essentially fewer options for resistance management for ITNs. Consequently, there is now an intensive effort to identify new insecticidal compounds for use in malaria control (Zaim and Guillet, 2002; Hemingway et al., 2006; Ranson, 2017).

Recently, new insecticides have been developed for vector control, including chlorfenapyr and clothianidin. When chlorfenapyr is activated by mixed-function oxidases, it is converted to a toxic form that uncouples oxidative phosphorylation in the mitochondria, resulting in disruption of energy production and subsequent death of the organism (Raghavendra et al., 2011; Oxborough et al., 2015). Its novel mode of action makes it unlikely to express any cross-resistance to standard neurotoxic insecticides. On the other hand, clothianidin is a neonicotinoid class insecticide commonly used against sucking agricultural pests (Ngufor et al., 2017; Thany, 2023). The compound acts on the nicotinic acetylcholine receptor (nAChR) in the insect central nervous system (Dagg et al., 2019; Thany, 2023). Both chlorfenapyr and clothianidin have been formulated for vector control and pre-qualified by the World Health Organization (WHO, 2023). Chlorfenapyr combined with alpha-cypermethrin into Interceptor® G2 long-lasting insecticidal nets (LLINs), has been found to be effective against pyrethroid-resistant malaria vectors (Mosha et al., 2022; Accrombessi et al., 2023). In addition, IRS chlorfenapyr shows potential to significantly improve the control of malaria transmission in areas with pyrethroid-resistant vectors compared to pyrethroid IRS or the mixture (Ngufor et al., 2017) and provides moderate but prolonged control of pyrethroid-resistant malaria vectors (Ngufor et al., 2020). Clothianidin has been developed as an IRS formulation by Sumitomo (SumiShield® 50WG) and Bayer (Fludora™ Fusion WP-SB) and provides an exceptionally good residual activity on all tested wall surfaces when compared to the residual activity of many other commercial insecticidal products used for IRS (Agossa et al., 2018; Kweka et al., 2018; Ngwej et al., 2019).

Although novel chemistry provides room for several resistance management strategies including rotation and insecticide mixture application, new insecticides may not provide a long-lasting and sustainable response to resistance if they are not judiciously deployed and accompanied by resistance monitoring. There is already a report of possible resistance against chlorfenapyr in Cameroon, DRC and Ghana (Tchouakui et al., 2023). Likewise, several studies have reported that, despite being effective against malaria vectors in the majority of sub-Saharan African countries, there are already reports on the reduced efficacy of clothianidin (Oxborough et al., 2019; Fouet et al., 2023). Therefore, judicious application together with early identification of resistance mechanisms to these insecticides and subsequent development of reliable resistance monitoring tools is of paramount importance to prolong and sustain efficacy (Black et al., 2008). Early resistance detection will guide responsible programmes such as the National Malarial Control Programme of Tanzania to take appropriate measures to prevent the spread and effect of resistance. While new generation LLINs (Interceptor G2, Olyset Plus, PermaNet 3.0) and IRS (Fludora Fusion) have already been rolled out in large programmes for community use, there are no markers of resistance to these insecticides to aid monitoring of resistance development against new insecticides incorporated in these tools.

The ambition to solve the insecticide resistance problem should therefore go beyond investing in insecticides with new modes of action, but to consider developing insecticides or strategies that would become resilient to mosquito evolution, either by targeting adult mosquitoes (Read et al., 2009), use of diverse insecticides in space and time, or insecticide mixtures (Curtis et al., 1998; Kolaczinski and Curtis, 2004; Nauen, 2007; Koella et al., 2009).

This study reports laboratory screening of new chemistry (clothianidin, chlorfenapyr and alpha-cypermethrin) against *Anopheles gambiae* (*sensu stricto*) Muleba-Kis strain as an early insecticide resistance development indicator upon exposure to sub-lethal doses, a proxy to natural encounter with vector control tools. The present study investigated trends in mortality (phenotypic resistance) of the Muleba-Kis strain following several exposures of larvae and adults to sub-lethal doses of chlorfenapyr, clothianidin and alpha-cypermethrin under laboratory conditions.

2. Materials and methods

2.1. Mosquito colony characterization

The *An. gambiae* Muleba-Kis strain was established at our insectary at the Kilimanjaro Christian Medical University College, Tanzania, in 2012 by crossing male mosquitoes from the F_1 generation of wild pyrethroid-

resistant mosquitoes with susceptible female *An. gambiae* (*s.s.*) Kisumu laboratory strain. The hybrid was subsequently selected routinely using a pyrethroid insecticide solution at the larval stage. The strain is periodically tested for phenotypic and genotypic resistance and monitored for adult weight and wing length. Details on the establishment and maintenance of this strain are given in a previous work (Azizi et al., 2021). In the present experiment, this strain was taken to a separate room in the insectary and divided into four colonies: CFP-selected adults, CFP-selected larvae, CTD-selected adults and CTD-selected larvae. These colonies were treated separately, with the "adult-selected colonies" selected at the adult stage by either CFP or CTD. On the other hand, the larvae-selected colonies were selected at the larval stage by CFP or CTD. These colonies were reared at a temperature of 20–35 °C, relative humidity of 60–90%, and a natural 12:12 h L:D photoperiod, and were provided with a guinea pig for blood-feeding and filter paper medium for egg-laying to propagate the colonies to the next generations.

Prior to selection experiments, the test system, *An. gambiae* (*s.s.*) Muleba-Kis strain, was characterized through cone bioassays, synergy bioassays, and susceptibility bottle bioassays.

2.1.1. Cone bioassays

The cone assays were carried out against alpha-cypermethrin and permethrin standard nets following the WHO guidelines (WHO, 2013).

2.1.2. Synergist-insecticide WHO tube bioassays

In a separate experiment, a synergist assay was carried out with piperonyl butoxide (PBO), a chemical that inhibits p450 enzymes, to assess the role of elevated mixed-function oxidases in pyrethroid-resistant *An. gambiae* (*s.s.*) Muleba-Kis strain. One hundred and twenty, 2–5-day-old female mosquitoes were pre-exposed to 4% piperonyl butoxide (PBO) in 4 replicates (25 mosquitoes per tube) for 1 h pre-exposure and then followed by 1-h exposure to permethrin papers (0.75%), following the WHO guidelines (WHO, 2016). Mortality was recorded at 24 h post-exposure. In brief, mosquitoes were pre-exposed to either untreated papers or 4% PBO papers for 1 h at a temperature of 27 ± 2 °C and a relative humidity of 70 ± 10% during exposure and post-exposure time. The two batches of mosquitoes were then transferred to holding cages for 60 min before being exposed for 1 h to permethrin papers (0.75%) and PY-control papers. After exposure, the mosquitoes were transferred into holding cups and provided with 10% glucose-soaked cotton pads. Mortality was recorded 24 h post-exposure.

2.1.3. Susceptibility bottle bioassays

Insecticide susceptibility bioassays were performed with Generation 0, following the WHO guidelines (WHO, 2022a). Bioassays were carried out using three insecticides, i.e. alpha-cypermethrin (12.5 µg/ml), chlorfenapyr (100 µg/ml), and an adjusted diagnostic concentration for clothianidin (90 µg/ml). Assays were conducted at a temperature of 25 ± 2 °C and a relative humidity of 80 ± 10%. Each type of insecticide bioassay was performed in 5 replicates, including one as a control. Twenty to 25, 2–5-day-old female, unfed mosquitoes were tested, constituting a sample size of 100–125 mosquitoes for each insecticide. Tested mosquitoes were monitored for mortality at 24 h post-exposure for alpha-cypermethin and at 24 h and 72 h post-exposure for chlorfenapyr and clothianidin.

2.2. Insecticide concentration optimization against larvae

The larval selection was preceded by optimization of insecticide concentration as follows. Target concentrations resulting in moderate mortality (34–66%) were investigated by exposing 5 replicates of 100 mosquito larvae separately at each concentration and recording mortality at 72 h post-exposure. For chlorfenapyr, the concentrations used were 0.3 mg/ml, 0.2 mg/ml, 0.005 mg/ml, 0.0025 mg/ml and 0.0015 mg/ml while those of clothianidin were 0.3 mg/ml, 0.1 mg/ml, 0.08 mg/ml, 0.06 mg/ml, 0.05 mg/ml and 0.015 mg/ml. After testing the first concentration and recording the 72-h mortality, mortality was 100%, hence the next concentration (lower than the starting/previous concentration) was tested and mortality was observed. The process was repeated until a concentration that resulted in 72-h mortality in the range of 34–66% was achieved. The choice of starting concentrations was based on previous starting doses for pyrethroid dose optimization for this colony, where the starting concentration was 0.3 mg/ml. The previously optimized concentration (0.08 mg/ml) for alpha-cypermethrin was used for the adult selection. For adult mosquito selection, a dose quarter of the discrimination concentration was selected for both chlorfenapyr and clothianidin (at 25.0 and 22.5 µg/bottle respectively), while 12.5 µg/bottle was used for the alpha-cypermethrin. Normal procedures for running bottle bioassays were used (WHO, 2022b), except for adjusted diagnostic dosage for clothianidin at 90 µg/bottle which seems to work well with our local populations (Tungu et al., 2022). The use of methyl esters of rapeseed oil (MERO) was avoided due to reports on its influence on insecticide performance which could hinder detection of resistance (Ashu et al., 2023).

2.3. Larvae selection

At each of the selected generations (1st, 3rd, 5th, 7th and 9th), bowls with approximately 200 3rd-to 4th-instar larvae of *An. gambiae* (*s.s.*) Muleba-Kis were selected using the following concentration, adopting a modified method by Shidrawi (1957) as explained in our previous study (Azizi et al., 2021): (i) chlorfenapyr (CFP) (0.0015 mg/ml); and (ii) clothianidin (CTD) (0.015 mg/ml).

In brief, for each glass bowl containing 1 litre of tap water at 27–32 °C, 1 ml of insecticide solution (or equivalent volume of water in the control bowl) was added and stirred for 1 min using a Pasteur pipette, and then left for 10 min. Around 200 larvae were then transferred into each glass bowl with the dissolved insecticide solution. A small amount of larval food was added, and the larvae were left for 24 h in the selection bowl. The larvae were sieved and rinsed with 500 ml tap water (maintained at a temperature of 27–32 °C) and returned to their original plastic bowls, and reared, while the dead larvae were removed. Only the selected survivors were propagated into the next generations for subsequent selections.

2.4. Adult selection

In a separate group, adult mosquitoes were exposed to up to 10 generations to chlorfenapyr (25 µg/bottle) and clothianidin (22.5 µg/bottle). In a distinct group, adult mosquitoes were exposed to up to 4 generations to alpha-cypermethrin (12.5 µg/bottle). At each stage of selection, all mosquitoes were fully selected by exposing batches of 20 mosquitoes/bottle until the whole cage was selected.

2.5. Susceptibility bioassays post-selection

Susceptibility bioassays were conducted at 5th, 7th and 10th generations for each population category: CFP adult-selected, CFP larvae-selected, CTD adult-selected and CTD larvae-selected in accordance with the WHO guidelines (WHO, 2013). For the alpha-cypermethrin (ACM) adult-selected, susceptibility tests were conducted at 2nd and 4th generations. In each test, a total of between 80 and 101 mosquitoes were used (except on two occasions where 40 mosquitoes were used), against the discrimination concentrations of the insecticides that were used for the selection. Bioassays for the ACM were carried out using bottle bioassay at 12.5 µg/bottle (WHO, 2022b), and WHO tube using permethrin (0.75%), and alpha-cypermethrin (0.05%) (WHO, 2016). Bioassays for the CFP group were carried out using bottle bioassay at 100 µg/bottle (WHO, 2022b) while bioassays for the CTD group were carried out using bottle bioassay at an adjusted concentration of 90 µg/bottle (Tungu et al., 2022). The control bottles (treated with 1 ml of acetone) were run along with the insecticide-treated bottles. All

bioassays were conducted at a temperature of 25 ± 2 °C and a relative humidity of 80 ± 10%. Each bioassay was performed against a control (treatment without insecticide). Twenty to 25, 2–5-day-old female, blood-unfed mosquitoes were tested against each insecticide. Mortality was recorded at 24 h (ACM) or at 24 h and 72 h post-exposure for CFP and CTD. Phenotypic resistance of the selected colonies at the 5th, 7th and 10th generations were compared with phenotypic resistance at baseline (before selection).

2.6. Interpretation of experimental data

The WHO criteria were used to classify the resistance or susceptibility status of the tested mosquito populations (WHO, 2013), stating that: mortality < 90% is indicative of resistance, mortality levels from 90 to 97% are suggestive of probable resistance and need further investigation, and mortality ≥ 98% is indicative of susceptibility. The mortality of a test sample was calculated by summing the number of dead mosquitoes across all replicates (bottles/tubes). Percentage mortality was expressed as the total number of dead mosquitoes divided by the total number of tested mosquitoes multiplied by 100.

3. Results

3.1. Characterization of mosquitoes and optimization of selection concentrations

Results from the cone bioassays (against IG2 and Olyset LLINs), WHO tube bioassays (against permethrin with and without synergist), and bottle bioassays (against CFP and CTD) are indicated in Table 1.

For CFP, all concentrations resulted in higher mortality from 90 to 100% mortality except for the concentration of 0.0015 mg/ml which resulted in moderate mortality (60–70%) and was therefore chosen for the mosquito population selection.

For CTD, all concentrations resulted in higher mortality (90–100%) except for the concentration of 0.015 mg/ml which resulted in moderate mortality (40–50%) and was therefore chosen for the mosquito population selection.

The results of optimization tests with *An. gambiae* (*s.s.*) Muleba-Kis strain before selection are indicated in Table 2 (see Supplementary Table S1 for raw data).

3.2. Susceptibility bioassays post-selection

High mortality was observed throughout 10 generations for both larvae and adults that were selected against chlorfenapyr. The 24-h mortality for selected larvae and selected adults ranged from 92% to 100% and from 95% to 100%, respectively, while the 72-h mortality for both selected larvae and selected adults was similar, within the range of 98–100%. (Figs. 1 and 2; Supplementary Table S2).

Selection with clothianidin at the larval and adult stage showed variable mortality (24-h mortality of 18–76% and 72-h mortality of 30–91% for selected larvae and 24-h mortality of 23–83% and 72-h mortality of 40–87%% for selected adults) in the selected generations (5th, 7th, and 10th) (Figs. 3 and 4; Supplementary Table S2). Moreover, there were significant differences in mortality for Generation 5 and Generation 7 between selected adults and larvae. One explanation could be the small sample size for Generation 5 for adults (nearly twice as small; $n = 40$) and Generation 7 for larvae (twice and more as small; $n = 40$). In general, a gradual resistance increase to clothianidin was observed during Generations 5 and 7 following larval and adult selection which was followed by an abrupt decrease of resistance in Generation 10.

In a distinct group, adult mosquitoes that were selected for only up to 4 generations with alpha-cypermethrin (12.5 µg/bottle) showed high resistance (24-h mortality of 23–28%) (Fig. 5; Supplementary Table S2). Only three generations (0, 2nd and 4th) are shown here as the study (adult mosquito selection with pyrethroids) was still ongoing by the time we were concluding the CFP and CTD selection study.

Table 1
Characterization for the *An. gambiae* (*s.s.*) Muleba-Kis strain.

Test	Insecticide	Concentration	Mortality (%)	95% CI (%)
Cone (IG2 LLIN)	ACM and chlorfenapyr (CFP)	100 and 200 mg/m^2	16	20–46
Cone (Olyset LLIN)	Permethrin	800 mg/m^2	5	3–22
WHO tube assay	Permethrin	0.75%	49	39–59
WHO tube assay	PBO and permethrin	4%	100	93–100
Bottle bioassay	ACM	12.5 µg/ml	53	43–63
Bottle bioassay	Chlorfenapyr (CFP)	100 µg/ml	99	97–101
Bottle bioassay	Clothianidin (CTD)	90 µg/ml	100	96–100

Abbreviations: ACM, alpha-cypermethrin; PBO, piperonyl butoxide; CI, confidence interval.

Table 2
Optimization of insecticide dosage for larvae selection.

Insecticide	Concentration (mg/ml)	No. exposed	Average 72-h mortality (%)
Chlorfenapyr	0.3	500	100
	0.2	500	99.8
	0.005	500	99.0
	0.0025	500	84.8
	0.0015	500	63.8[a]
Clothianidin	0.3	500	100
	0.1	500	99.2
	0.08	500	100
	0.06	500	86.4
	0.05	500	73.2
	0.015	500	43.8[a]

[a] Target concentrations resulting in moderate mortality (34–66%).

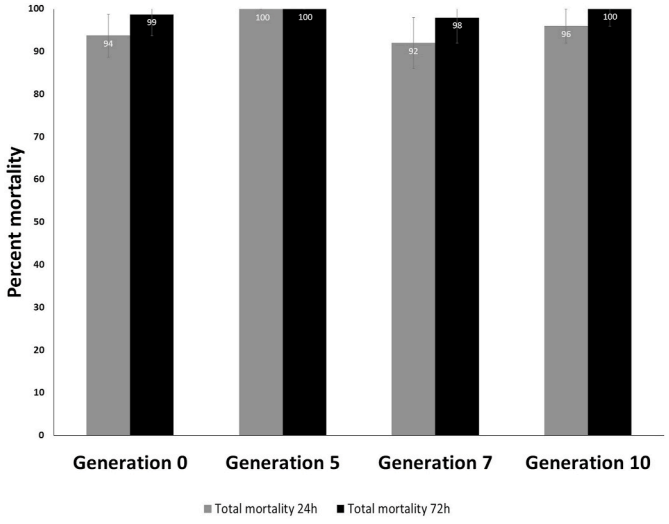

Fig. 1. The mortality of *An. gambiae* (*s.s.*) Muleba-Kis strain from chlorfenapyr larvae selection against 100 µg/bottle chlorfenapyr. Bars indicate percent mortality for sample sizes of 80 mosquitoes for Generations 0 and 5, and of 100 mosquitoes for Generations 7 and 10. Error bars represent 95% confidence intervals.

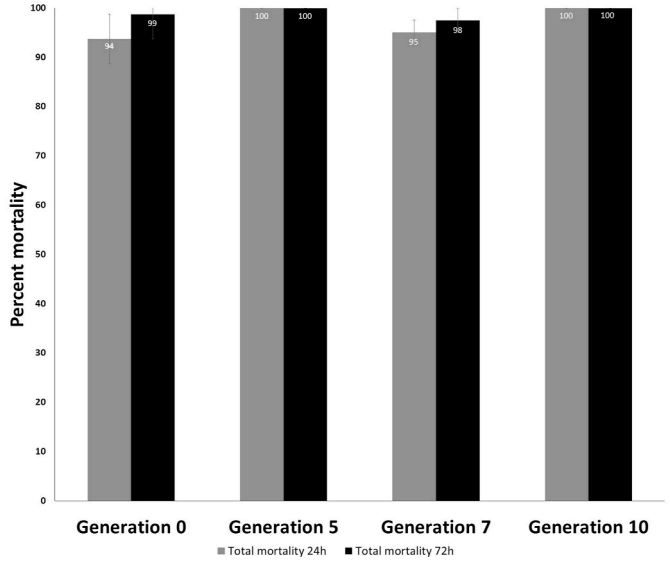

Fig. 2. The mortality of *An. gambiae* (*s.s.*) Muleba-Kis strain from chlorfenapyr adult selection against 100 μg/bottle chlorfenapyr. Bars indicate percent mortality for sample sizes of 80 mosquitoes for Generations 0 and 5, 81 mosquitoes for Generation 7, and 104 mosquitoes for Generation 10. Error bars represent 95% confidence intervals.

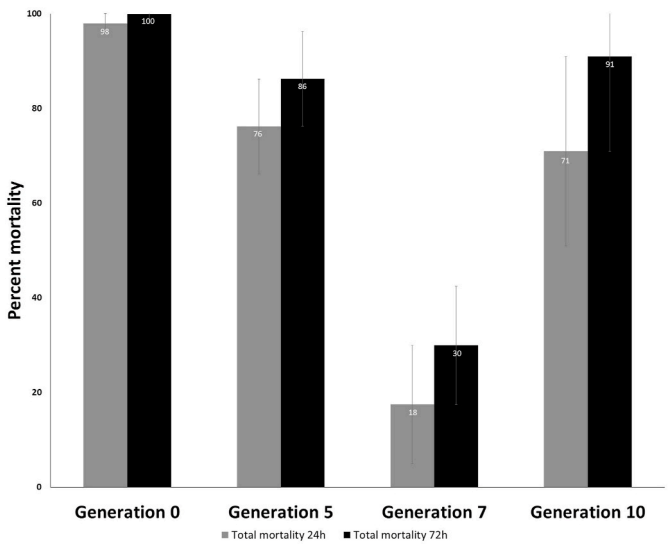

Fig. 3. The mortality of *An. gambiae* (*s.s.*) Muleba-Kis strain from clothianidin larvae selection against 90 μg/bottle clothianidin. Bars indicate percent mortality for sample sizes of 80 mosquitoes for Generations 0 and 5, 40 mosquitoes for Generation 7, and 100 mosquitoes for Generation 10. Error bars represent 95% confidence intervals.

4. Discussion

Recent development and the introduction of new insecticides for malaria vector control, have already led to recommendations of new commercial vector control formulations that are pre-qualified by the WHO (WHO, 2022c). Chlorfenapyr is combined with alpha-cypermethrin into Interceptor® G2 LLIN, produced by BASF, and it is also under evaluation as an IRS product (Sylando® 240SC). Clothianidin has been produced as an IRS formulation in SumiShield™50 WG, Klypson 500 WG, Fludora™ Fusion and 2GARD (WHO, 2022c). Although novel chemistries provide room for several resistance management strategies, still mosquitoes have proven in previous studies to develop resistance against several insecticides that were initially regarded as "new" and "effective", but consequently undermined by the evolution of resistance.

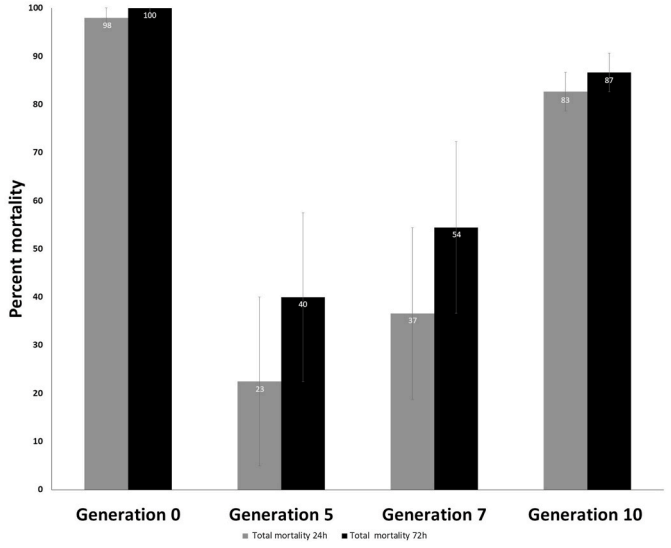

Fig. 4. The mortality of *An. gambiae* (*s.s.*) Muleba-Kis strain from clothianidin adult selection against 90 μg/bottle clothianidin. Bars indicate percent mortality for sample sizes of 80 mosquitoes for Generation 0, 40 mosquitoes for Generation 5, 101 mosquitoes for Generation 7, and 75 mosquitoes for Generation 10. Error bars represent 95% confidence intervals.

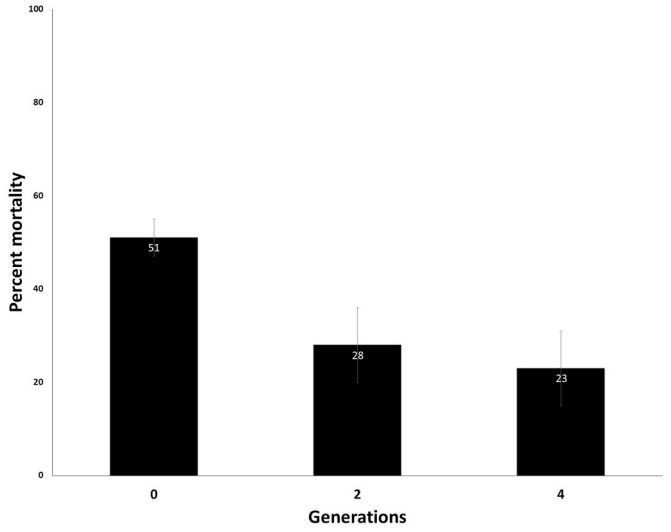

Fig. 5. The mortality of *An. gambiae* (*s.s.*) Muleba-Kis strain from alpha-cypermethrin adult selection against 12.5 μg/bottle alpha-cypermethrin. Bars indicate percent mortality for sample sizes of 100 mosquitoes for Generation 0 and 80 mosquitoes for Generations 2 and 4. Error bars represent 95% confidence intervals.

This study was carried out to investigate the possible development of resistance in mosquito populations after several exposures to insecticidal selection pressure at the larval or adult stage. This was done by using chlorfenapyr, clothianidin, and alpha-cypermethrin (ACM), where larvae or adults were selected using sub-lethal doses every one generation to determine whether adult mosquitoes in the subsequent generations would develop resistance against diagnostic concentrations.

At the diagnostic dose of 100 μg/bottle chlorfenapyr was found to cause persistently high mortality in the adult mosquitoes before and after being selected up to Generation 10 at both larval and adult stage.

Laboratory selection with chlorfenapyr did not indicate a differential effect on the resistance development between mosquitoes selected at adult and larval stages. This could be attributed to the underlying resistance mechanism in the used mosquitoes, known for its overexpressed multiple P450 genes including CYP6M2, CYP6Z3, CYP6P3, CYP6P4, CYP6AA1 and CYP9K1 as reported by Matowo et al. (2022). Therefore, it is likely the observed high mortalities for chlorfenapyr are due to the metabolism of chlorfenapyr by P450 enzymes. Chlorfenapyr is a pro-insecticide that requires P450 activation to produce tralopyril (the toxic metabolite) and other bioactive metabolites. Pyrethroid resistance is often associated with elevated levels of chemoprotective P450s with broad substrate specificity, which could influence chlorfenapyr activity. In the recent report by Yunta et al. (2023), chlorfenapyr was activated to tralopyril by *An. gambiae* CYP6P3, CYP9J5, and CYP9K1.

Testing the mosquitoes against new and 36 months-old chlorfenapyr-treated IG2 nets still indicates the efficacy of the insecticide against selected mosquito colonies (Azizi et al., 2023). Our result is consistent with several study findings, where chlorfenapyr was found to be effective against pyrethroid-resistant mosquitoes (Oxborough et al., 2015; N'Guessan et al., 2016). This is partly attributed to the fact that chlorfenapyr is a pro-insecticide requiring activation by cytochrome P450 monooxygenase enzymes (P450s), which commonly confers resistance to other insecticides in this mosquito strain (Azizi et al., 2021). However, a recent report from the Democratic Republic of Congo, Cameroon and Ghana, has documented chlorfenapyr resistance in *Anopheles* mosquito populations (Tchouakui et al., 2023), although the same study has reported high susceptibility to chlorfenapyr, including mosquitoes with multiple insecticide resistance mechanisms. The reported resistance to chlorfenapyr was attributed to the intensive use of various pesticides in agriculture (Tchouakui et al., 2023).

Based on the results of these laboratory selections, the potential for *Anopheles gambiae* (*s.s.*) to develop resistance to clothianidin does exist. Exposing *An. gambiae* (*s.s.*) Muleba-Kis strain larvae to sub-lethal concentration of clothianidin affected their subsequent tolerance to clothianidin, where mortality rates decreased from the parental colony to Generation 7. A similar pattern of resistance was observed with adult selection although much higher in the adult-selected arm compared to the larvae-selected arm. However, higher mortality rates were observed in Generation 7 than in Generation 5 although the difference was not significant. The differences between adults and larvae for Generation 5 and Generation 7 may be related to the small sample size tested for Generation 5 for adults and Generation 7 for larvae. However, sample size does not seem to be a problem since similar studies on pest resistance have recommended even lower sample sizes (Miller et al., 2010). Additionally, since bioassays were conducted using healthy and strong mosquitoes during these points and subsequent generations, where results still indicate resistance, the effect of sample size at one time point does not affect resistance interpretation. In both cases, partial restoration of susceptibility was observed with a 72-h mortality rate of about 90% in Generation 10. Similar findings have been reported recently by Thornton et al. (2020) where insecticide resistance selection and reversal were observed in two strains of *Ae. aegypti.* The increase in mortality rates in Generation 10 may be associated with negative fitness costs. Investigations to attribute the decrease in resistance to fitness cost or other factors were not within the scope of this study and there is a need for further study. Population cage assays using a laboratory lineage of *Ae. aegypti* resistant to pyrethroids due to *kdr* mutation presented deleterious effects where the *kdr* allele severely decreased from 75% to almost zero along 15 generations (Brito et al., 2013).

Clothianidin has been recommended by the WHO to be added to the current mainstays of mosquito control through IRS (WHO, 2017). Despite its effectiveness against malaria vectors in the majority of sub-Saharan African countries, there are already reports on the reduced efficacy of clothianidin (Oxborough et al., 2019). In the present study, selection at the adult and larval stages against sub-lethal concentrations of CTD has resulted in varying mortality rates, indicating a possible insecticide resistance development within 10 generations. Similar findings on the reduced potency after exposure to clothianidin-based IRS (SumiShield® 50WG) in *An. gambiae* adults collected from an area where neonicotinoids (acetamiprid and imidacloprid) are being used as agricultural pesticides were reported by Fouet et al. (2023). Following adult selection, the development of pyrethroid resistance in the *An. gambiae* (*s.s.*) Muleba-Kis strain was faster than that of chlorfenapyr and clothianidin. These findings are consistent with previous studies, where pyrethroids were used for adult selection (Williams et al., 2019; Machani et al., 2020).

The heavy use of pesticides in agricultural areas often leads to the contamination of nearby mosquito larval habitats, creating selection pressure that can affect the insecticide sensitivity of mosquito larvae and adults. Clothianidin has been used for a long time in agriculture to kill crop pests and it is most likely that the African malaria vectors are developing resistance to clothianidin through selection pressure in their larval habitats. In a recent study by Sadia et al. (2022), *An. gambiae* larvae were exposed to a sub-lethal dose of a mixture of agrochemical pesticides used in a highly active agricultural area on the Ivory Coast, and then monitored for clothianidin susceptibility. Bioassays revealed a significantly increased tolerance of adult females to clothianidin (2.5-fold) and Fludora Fusion mixture (2.2-fold) following larval exposure to agrochemicals. This suggests that although the complex interactions between the use of agrochemicals and vector control insecticides are difficult to interpret in the field, they still must be considered in the context of insecticide resistance management ITNs.

In this study, only selection with clothianidin induced resistance in the test mosquitoes within 10 generations. In a parallel experiment, the same mosquito strain (Muleba-Kis) developed high resistance within just four generations after selection with alpha-cypermethrin, similar to the observation made by Thornton et al. (2020). Since the test mosquitoes (Muleba-Kis strain) were already resistant (*kdr* and metabolic) to pyrethroids, subsequent selection with chlorfenapyr/clothianidin is a proxy to the field situation, where mosquito larvae/adults are exposed to a mixture of insecticides simultaneously. In such a scenario, it is expected that prior exposures to sub-lethal concentrations of the same or different insecticide class would induce resistance against the same or different insecticides (Opiyo et al., 2021; Yunta et al., 2023). This is particularly important as pyrethroid resistance is widespread, raising concern on the possibilities of cross-resistance between pyrethroids and other insecticides. Although this study has revealed the occurrence of resistance to clothianidin just within 10 generations with selection, it is difficult to extrapolate the results directly to the field environment where other factors such as the type of insecticide contaminants and the level of concentration or frequency of exposure will affect the onset of resistance. Nevertheless, it is important to note that these findings anticipate the emergence of resistance for clothianidin in the field earlier relative to chlorfenapyr.

Owing to its fast reproduction and high number of offspring, mosquitoes can rapidly adapt to selection pressure, such as sub-lethal exposure to insecticides. This also provides avenues for research experiments to study resistance evolution from artificial selection in the laboratory (Shidrawi, 1957; Williams et al., 2019; Machani et al., 2020; Azizi et al., 2021). Early knowledge of resistance patterns to a particular insecticide is necessary to prepare strategies for resistance management.

5. Conclusions

Subsequent exposure of *An. gambiae* (*s.s.*) Muleba-Kis larvae and adults to sub-lethal dose of chlorfenapyr did not induce any resistance to chlorfenapyr. However, based on laboratory selection results, the potential for *Anopheles gambiae* (*s.s.*) to develop resistance to clothianidin does exist. Further studies are needed to investigate the effect of selection beyond 10 generations and underlying mechanisms for observed tolerance against clothianidin. Finally, active surveillance and monitoring of insecticide resistance to chlorfenapyr and clothianidin in

malaria vectors is recommended to inform insecticide management strategies including rotational strategies that are based on the rotation over time of two or preferably more insecticide classes with different modes of action.

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Ethical approval

This study obtained ethical clearance from the Institutional Review Board of the Kilimanjaro Christian Medical University College (Research Ethical Clearance Certificate No. 2512), and is also part of the large ongoing research programme that was reviewed and approved by the Tanzania National Institute for Medical Research (NIMR/HQ/R.8c/Vol.1/554).

CRediT authorship contribution statement

Salum Azizi: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Njelembo J. Mbewe:** Conceptualization, Writing – review & editing, Formal analysis. **Hosiana Mo:** Conceptualization, Methodology, Writing – review & editing. **Felista Edward:** Conceptualization, Methodology, Writing – review & editing. **Godwin Sumari:** Methodology, Writing – review & editing. **Silvia Mwacha:** Methodology, Writing – review & editing. **Agness Msapalla:** Methodology, Writing – review & editing. **Benson Mawa:** Writing – review & editing. **Franklin Mosha:** Conceptualization, Funding acquisition, Project administration. **Johnson Matowo:** Conceptualization, Methodology, Writing – review & editing. All authors read and approved the final manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets generated and analyzed to support the conclusions of this article are provided within the article and its supplementary files.

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Appendix A. Supplementary data

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