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Multiple insecticide resistance in *Anopheles funestus* from Mopeia, Central Mozambique

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Abstract

Background The main malaria vector control methods implemented in Mozambique are insecticide-treated nets (ITN's) and indoor residual spraying (IRS). These insecticide-based interventions are currently threatened by the rapidly spreading insecticide resistance in several major malaria vectors. Monitoring of insecticide resistance is necessary to inform the selection of insecticides by control programmes. This study describes the insecticide resistance profiles of the main malaria vector, *Anopheles funestus* sensu lato. in Mopeia district, a malaria holoendemic area of the Zambezia province of Mozambique.

Methods *Anopheles* adults and larvae were collected from 15 sentinel sites across the district between October 2021 and September 2022. Wild-caught, unfed female adults were collected using CDC-light traps and pooled over three days before exposure to the test insecticide. For mosquitoes collected as larvae, F0 adults aged 3–5 days post-emergence were used for insecticide susceptibility testing. Resistance to bendiocarb, DDT, deltamethrin and pirimiphos-methyl was evaluated using the standard WHO tube bioassay. The mechanism of resistance was probed using the PBO (piperonyl butoxide) synergistic bioassay. The presence and frequency of different genetic mutations associated with insecticide resistance was assessed using polymerase chain reaction, including A296S-Rdl, L119F-GSTe2 and 6.5 kb SV (structural variation) insertion.

Results A total of 1349 female *Anopheles* mosquitoes (controls included) were used for susceptibility tests with discriminating insecticide concentrations. Phenotypic resistance to bendiocarb, DDT, deltamethrin and pirimiphos-methyl was observed, with 37%, 79%, 14% and 67% mortality rate respectively. Pre-exposure to PBO partially restored susceptibility to deltamethrin to a mortality rate of 80%. The frequency of the insecticide resistance mutations was 0.49, 0.05 and 0.92, for A296S-Rdl, L119F-GSTe2 and 6.5 kb SV insertion, respectively.

Conclusion Malaria vectors in Mopeia exhibit resistance to all four major public health insecticide classes: pyrethroids, organophosphates, organochlorides and carbamates. This highlights the urgent need to adopt new insecticide classes for vector control interventions. The partial restoration of susceptibility by PBO suggests resistance is being driven by various mechanisms including the involvement of metabolic resistance through cytochrome P450 monooxygenase enzymes and glutathione S-transferases.

Keywords Insecticide resistance, *Anopheles funestus*., Mozambique, Dieldrin

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Background

Despite intense global and national efforts to control and eliminate malaria, the disease remains a major public health challenge in Mozambique and is the leading cause of death in children under five years of age [1]. Malaria is endemic across Mozambique, with the entire population of approximately 31 million at risk. However, prevalence is highly heterogenous—ranging from less than 1% in the South to as high as 55% in the North [1, 2]. Mozambique is a member of the Elimination 8 (E8) initiative, a regional collaboration aimed at accelerating malaria elimination in Southern Africa [3]. The E8 focuses on eliminating malaria in the frontline, low transmission countries while supporting neighbouring second line, moderate to high transmission countries such as Mozambique, to also reach elimination [3, 4]. The emergence and spread of resistance in malaria vectors within Mozambique directly influence the success of elimination strategies in region.

In Mozambique, the primary malaria vector control strategies are indoor residual spraying (IRS) and insecticide-treated nets (ITNs). Both rely on insecticides and are threatened by rapid emergence and spread of resistance. Until recently, the World Health Organization (WHO) approved only four classes of insecticides for malaria vector control: pyrethroids, organophosphates, organochlorides and carbamates. However, the widespread development of resistance to these insecticides in main malaria vectors has necessitated the development of new alternative insecticides. For ITNs, chlorofenapyr (a pyrrole) and pyriproxyfen (an insect growth regulator) have been added to the list of WHO prequalified insecticides and are now used in third-generation dual-active ITNs to address pyrethroid resistance [5]. For IRS, new options include clothianidin (a neonicotinoid) and broflanilide (a meta-diamide) [6].

The selection of insecticides, as well as the deployment and combination strategy at national and sub-national level, primarily depends mainly on the susceptibility of local vectors to the active ingredient, the availability of funding, and the intensity of malaria transmission [7]. In the Zambezia province, where Mopeia district is located, IRS has been implemented in combination with ITNs since 2007 [8] showing a proven incremental impact [9, 10]. The U.S. President's Malaria Initiative (PMI) has supported IRS campaigns in the province in collaboration with the National Malaria Control Programme (NMCP) [8].

To date only pyrethroid-based ITNs have been distributed in the province [11]. In the case of IRS, multiple classes of insecticides have been used in the province (Fig. 1). Until 2009, DDT was used for IRS. A shift was made to pyrethroids in 2010 due to insecticide resistance concerns as well as increased DDT levels in the environment [12]. The use of pyrethroids continued until 2015, when a transition to organophosphates (Actellic CS 300) was made due to development of resistance to pyrethroids [13, 14]. In 2017, the WHO approved the neonicotinoid insecticide, clothianidin for use for IRS [15]. With the introduction of a new insecticide product to the market and as part of efforts to manage resistance, a transition from organophospahtes to neonicotinoids (SumiShield 50WG) was made in 2018. Neonicotinoids were used until 2020, either as the single formulation of clothianidin (SumiShield 50WG) or mixed formulation of clothianidin and deltamethrin (Fludora Fusion) [16–18]. Following a recommendation for rotation of different classes of insecticides [19], in 2021, Mozambique adopted a two year rotation plan that prompted the use organophosphate (Actellic 300CS) and carbamate (Bendiocarb 80WP-SB, Ficam) [20, 21]. Since 2007 to date, Mopeia district has had IRS implemented, with the exception of

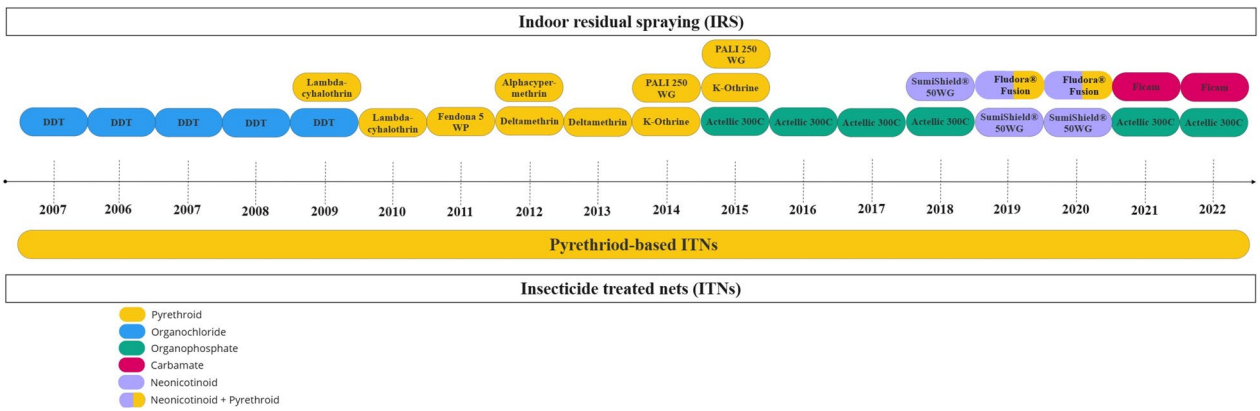

Fig. 1 History of Insecticide based interventions for malaria vector control in Zambezia province

2012, 2013 and 2015, where the district was excluded in the annual IRS campaign [16].

With Mopeia having a history of extensive use of insecticides for malaria vector control, it is important to understand the insecticide resistance profile of the malaria vectors. This is not only key for informing the selection of insecticides and managing insecticide resistance but also to understand the background and context for non-insecticide-based interventions implemented in the area. This study evaluated the insecticide resistance profiles, and possible underlying mechanisms, in the main malaria vector, *Anopheles funestus* sensu lato (*s.l.*) in Mopeia district. The study was done in the context of the BOHEMIA (Broad One-Health Endectocide-based Malaria intervention in Africa), a clinical trial evaluating the impact of ivermectin mass drug administration to humans and animals on malaria transmission implemented in Mopeia [22].

Methods

Study area and setting

The study was conducted in Mopeia District, Zambezia Province. The study area has previously been described [23]. The study was done in the context of the BOHEMIA trial, a three-arm cluster randomized controlled trial [22]. Mosquitoes were collected from 15 sentinel clusters, with five clusters belonging to each of the three treatment arms: ivermectin MDA to humans only arm, ivermectin MDA to humans and livestock arm and albendazole MDA to humans (control) arm (Fig. 2).

Mosquito sampling

Anopheles adults and larvae were collected from October 2021 to September 2022. Host-seeking adults were collected monthly indoor and outdoor using CDC light traps for three consecutive days, twice a month on two non-consecutive weeks. Unfed female adults were selected and maintained in the insectary at 26 °C ± 2 °C and 70% ± 10 relative humidity and fed with 10% sucrose ad libitum. Mosquitoes from the 3-collection-days were pooled to attain enough numbers for the insecticide susceptibility bioassays. Larval collections were done from various habitats in the study area. Larvae habitats were searched near houses which yielded high densities of adult mosquitoes by light trap collection. The larvae were maintained in their natural habitat water in an outdoor

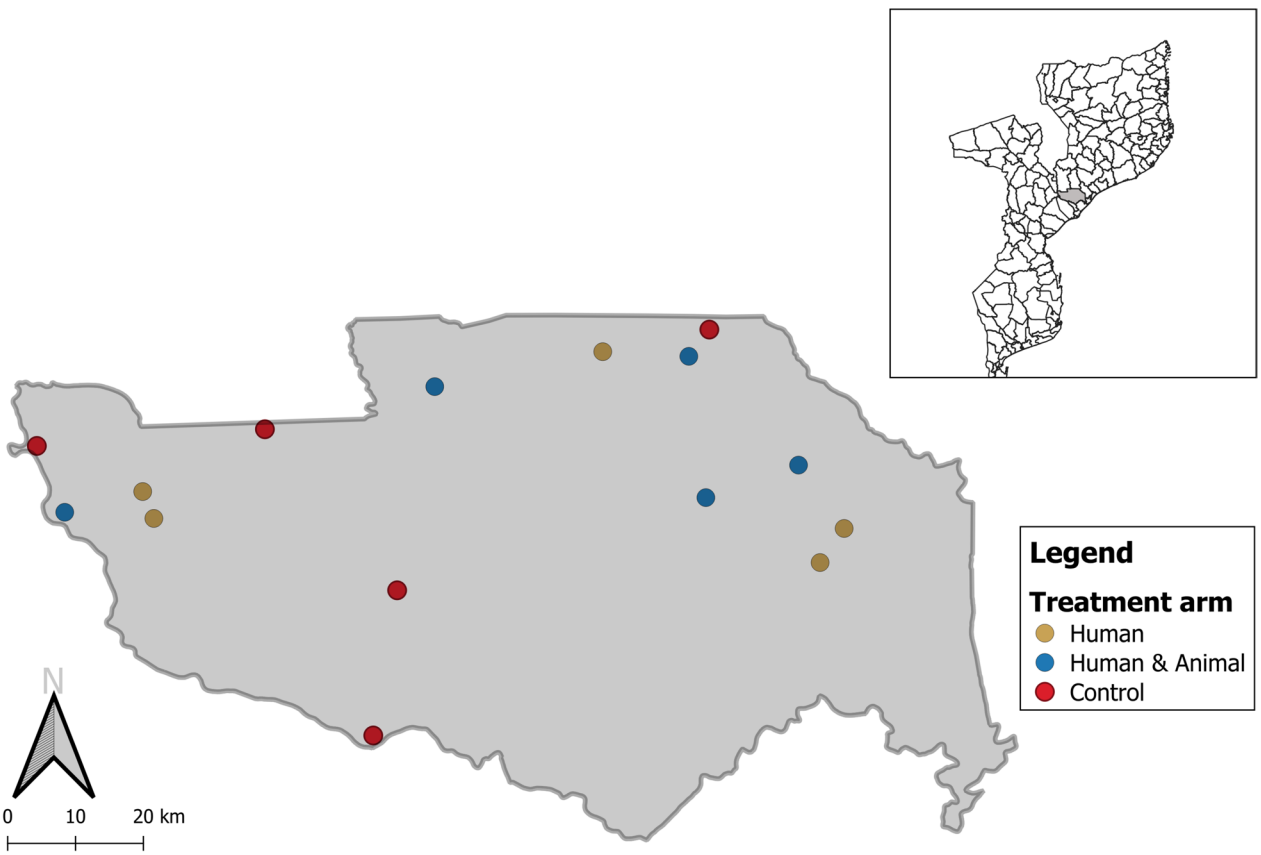

Fig. 2 Map of the study area showing the sentinel clusters and their assigned treatment arm where mosquitoes were collected

insectary at natural ambient conditions. The larvae were fed with ground fish food until pupation. Pupae were collected and placed in paper cups to obtain F0 adults. The emerging adults were maintained on 10% sucrose and used for insecticide susceptibility assays at the age of 3–5 days old.

Insecticide resistance phenotype (susceptibility and synergist bioassays)

In both the wild-collected adults and the F0 adults, mosquitoes were pooled by treatment arm for the insecticide susceptibility bioassays. The bioassays were carried out using the WHO insecticide susceptibility tube-test procedure [24]. Test papers impregnated with the WHO-recommended discriminating concentration of deltamethrin (0.05%), DDT (4%), pirimiphos-methyl (0.25%) and bendiocarb (0.1%) were used. The quality of the test paper was checked using a laboratory susceptible colony, *Anopheles arabiensis* KGB strain. Mosquitoes assigned to controls were exposed to untreated control papers.

The insecticide susceptibility bioassays were done using WHO tube tests and following standard guidelines [24]. Testing was done using batches of 15–25 female mosquitoes per tube, each batch was exposed for 1 h to the insecticide-impregnated papers, or control. After one hour of exposure, the mosquitoes were transferred to holding tubes and held for 24 h with access to 10% sucrose. Mortality was scored at 24 h post-exposure and resistance status determined using the WHO criteria whereby they are classified as either: (1) susceptible—if recorded mortality is higher or equal to 98%; (2) possibly resistant—if recorded mortality is between 90 and 98%; or (3) confirmed resistant—if recorded mortality is below 90% [24]. As the mosquitoes were collected in the context of a three-arm cluster randomized trial, the susceptibility bioassays were also separated by treatment arm. A multivariable logistic regression model was conducted to evaluate the influence of treatment arm and source of adult mosquitoes (wild-collected adults or F0 adults from larvae collection) on mosquito mortality.

Involvement of potential metabolic resistance was evaluated by conducting a synergistic bioassay using PBO. This was done by pre-exposing the mosquitoes to PBO 4% for 1 h before they were exposed to papers impregnated with deltamethrin for 1 h (PBO + deltamethrin) [24]. Controls consisted of mosquitoes exposed to papers with PBO-only, deltamethrin-only, and untreated paper. Mortality was recorded at 24 h post-exposure. To evaluate the ability of PBO to restore mosquito susceptibility to pyrethroids, mortality in mosquitoes exposed to PBO + deltamethrin was compared to mortality in deltamethrin-only. The results were interpreted using the WHO criteria, whereby results were classified as either: (1) full restoration of susceptibility—if the recorded mortality in the PBO + deltamethrin group is higher or equal to 98%; (2) partial restoration of susceptibility—if the recorded mortality in the PBO + deltamethrin group is below 98% but at least 10% greater than the mean mortality in the deltamethrin-only group; or (3) no restoration of susceptibility—if mortality in the PBO + deltamethrin group is equal to or less than the mortality in the deltamethrin-only group [25].

After the bioassays, all mosquitoes were preserved by desiccation in silica gel in a 1.5-ml microcentrifuge tubes and shipped to KEMRI Wellcome Trust Research Programme (KWTRP), Kilifi, Kenya for further molecular analysis.

Species identification by MALDI-TOF MS

A subset of the mosquito samples (n = 210) from the bioassays were identified to species level by matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-TOF MS) [26]. Briefly, the head and thorax were divided in half, one half was used for species identification by MALDI-TOF MS while the other half was used for DNA extraction for DNA-based analysis. For MALDI-TOF MS, the head and thorax were homogenized in 70% formic acid and 50% acetonitrile and 1ul of the lysate used for spectra acquisition. Spectra acquisition was done using the Microflex machine (Bruker Daltonics) and the FlexControl software ver. 3.3.0 (Bruker Daltonics). The acquired spectra were queried using a reference library of Anopheline species using MALDI Biotyper Compass Explorer (Bruker Daltonics). Confidence in the identification was assessed using a threshold log-score value greater or equal to1.8. The species identification of all samples with a log score value less than 1.8 and those with no spectra (flat line), were confirmed by cocktail PCR assay described by Koekemoer et al. [27].

Determination of the *Rdl* mutation genotype

In *Anopheles*, resistance to dieldrin is determined through a point mutation on the dieldrin locus (*Rdl*) which codes for the γ-aminobutyric acid (GABA) receptor. The *Rdl* mutation was determined using polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) as described by Wondji et al. [28]. Briefly, the 255 bp fragment of the *Rdl* gene was amplified followed by digestion of the amplification product by HpyCH4V restriction enzyme. The restriction enzyme cleaves only the susceptible allele to generate two fragments: 117 and 138 bp allowing for the identification of resistant, susceptible and heterozygous genotypes [28].

DNA extracts of 189 mosquito samples (remaining half of head and thorax not used for MALDI-TOF) was

genotyped for the *Rdl* resistant mutation (Rdl^R) and frequency of the resistance genotype, Rdl^R calculated.

Evaluation of the 6.5 kb SV insertion

To evaluate for P450-based metabolic resistance, the 6.5 kb SV insertion was genotyped. This is a structural variation in the form of a 6545 base pairs insertion between two *P450* genes, *CYP6P9a* and *CYP6P9b* that is associated with overexpression of the two *P450* genes, subsequently *P450*-mediated insecticide resistance [29, 30]. The presence of the 6.5 kb SV insertion was determined using allele-specific polymerase chain reaction (AS-PCR) as described by Mugenzi et al. [30]. A 596-bp fragment is amplified in the presence of the insertion while a 266 bp fragment is amplified in the absence of the insertion.

Determination of the L119F-GSTe2 mutation genotype

To evaluate for glutathione-s-transferase epsilon 2 (*GSTe2*) based metabolic resistance, the L119F mutation was genotyped. The mutation is a single amino acid change in the *GSTe2* gene that confers DDT and pyrethroid resistance. The presence of the mutation was determined by AS-PCR that yields a 523 bp fragment for the resistant genotype, 312 bp fragment for the susceptible genotype and an 849 bp fragment common for all genotypes [31].

Results

Insecticide resistance phenotype

A total of 1,349 female *Anopheles* mosquitoes were tested for insecticide susceptibility. Of these, 870 were exposed to insecticides while 479 were exposed to the respective control. All tests met the inclusion criteria of mortality in the controls < 20%. Where mortality in the controls was more than 5%, it was corrected using Abbott's formula [24].

Phenotypic resistance to bendiocarb, DDT, deltamethrin and pirimiphos-methyl was observed with 37%, 79%, 14% and 67% mean mortality rate, respectively (Fig. 3). Resistance was observed across all the treatment arms (Table 1). The number of mosquitoes collected in the control/no intervention arm was significantly higher, thus the high number of mosquitoes used for the bioassay and enabling the testing of more insecticides. Though resistance was reported in all treatment arms, the logistic regression model revealed that the treatment arm was a significant predictor of mosquito mortality. A significantly higher mortality was observed in the human and animal treatment arm (odds ratio [OR] = 1.87, p = 0.01). The source of adult mosquitoes used for the bioassay (wild-collected adults or F0 adults from larvae collection) had no influence on mortality.

A total of 168 mosquitoes were used to perform two PBO-synergist bioassays. One test was excluded due to unexplained high mortality in the PBO-only treatment.

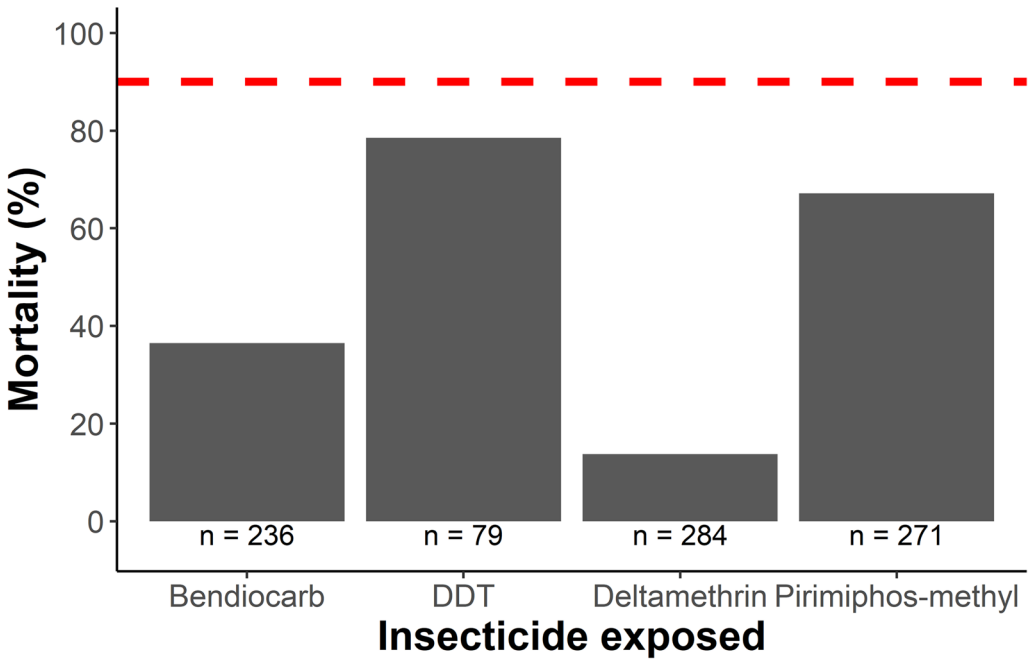

Fig. 3 Mortality of *An. funestus s.l.* 24 h post-exposure to discriminating concentrations of insecticides. Error bars represent the standard deviations and, red dotted line represent the WHO resistance and susceptibility threshold respectively

Table 1 Resistance status of *An. funestus s.l.* exposed to bendiocarb, DDT, deltamethrin and pirimiphos-methyl across different sentinel sites of the BOHEMIA trial study site (Mopeia, Zambezia Province, Mozambique)

Treatment arm	Insecticide	N	Mortality (95% CI)	Resistance status	SD
Control	Bendiocarb	139	29.5 (16.74–41.45)	Resistant	15.44
Control	DDT	79	78.48 (67.47–89.56)	Resistant	11.27
Control	Deltamethrin	166	16.87 (11.44–21.57)	Resistant	6.33
Control	Pirimiphos-methyl	85	68.24 (32.36–104.91)	Resistant	37.01
Human and animal	Bendiocarb	45	48.89 (46.48–51.14)	Resistant	1.68
Human and animal	Deltamethrin	54	37.04 (4.24–64.11)	Resistant	24.66
Human and animal	Pirimiphos-methyl	90	66.67 (58.79–74.95)	Resistant	8.25
Human	Bendiocarb	52	44.23 (19.75–88.02)	Resistant	30.17
Human	Deltamethrin	64	10.94 (6.15–16.58)	Resistant	4.61
Human	Pirimiphos-methyl	96	68.75 (55.94–81.77)	Resistant	13.18

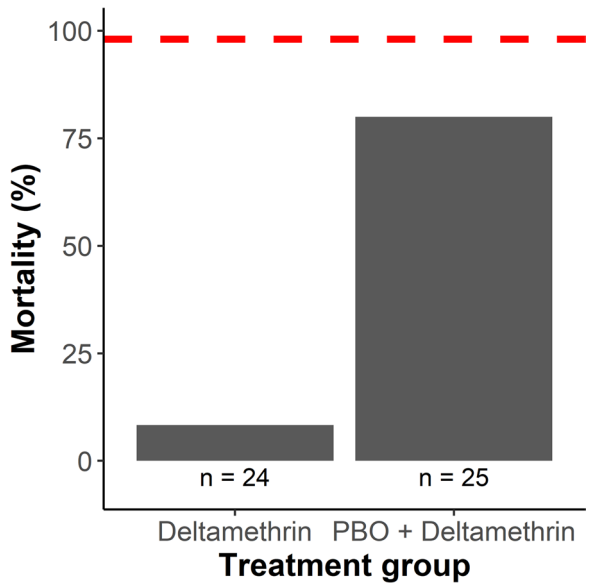

Fig. 4 Mortality of *An. funestus s.l.* after pre-exposure to PBO. Red dotted line represents the WHO confirmed resistance threshold

It is worth noting that all mosquitoes used in the PBO synergist bioassays were wild collected females of an unknown age. In the second bioassay, pre-exposure to PBO restored some susceptibility to deltamethrin with mortality increasing from 8% in deltamethrin-only group to 80% in deltamethrin + PBO group (Fig. 4). This partially restored susceptibility to deltamethrin suggests the involvement of monooxygenase enzymes in the observed deltamethrin resistance.

Species composition

A total of 210 *An. funestus s.l.* mosquitoes were analysed by MALDI-TOF MS to determine their species identity. One hundred and eighty-three mosquitoes were classified into either of the three species: *An. funestus* sensu stricto (*s.s.*) (170/210), *Anopheles rivulorum* (n = 3/210) and *Anopheles leesoni* (n = 1/210). The rest (n = 35/210) had inconclusive results due to either a low log-score value below the acceptable threshold of 1.8 (n = 27/36) or a flat line indicating that no spectra was acquired (n = 9/36). All the 36 samples with inconclusive results were subjected to molecular identification using the *An. funestus s.l.* cocktail PCR, where 19/36 samples were found to be *An. funestus s.s.* while the rest (17/36) did not amplify.

Table 2 Genotype and allele frequencies of *Rdl* mutation in *An. funestus s.l.* from Mopeia, Zambezia province, Mozambique

Species	SS	RR	RS	Rdl^R frequency
An. leesoni	0	0	1	0.5
An. rivulorum	0	0	3	0.5
An. funestus s.s	9	7	104	0.49
Overall	9 (7.26%)	7 (5.65%)	108 (87.10%)	0.49

Rdl insecticide resistance genotype

A total of 189 *An. funestus s.l.* samples were screened for the presence of *Rdl* resistance gene. Out of these, 145 successfully amplified the expected fragment of 255 bp. The 145 amplified samples were digested with the HpyCH4V restriction enzyme, 124 of which were genotyped as either susceptible (SS), resistant (RR) or heterozygous (RS). Overall, 7.26% (9/124) of the mosquitoes tested were homozygote susceptible (SS), 5.65% (7/124) were homozygote resistant (RR) and 87.10% (108/124) were heterozygote (RS) (Table 2). Overall, the frequency of the RdL^R allele was 0.49, with a frequency of 0.5 in *An. leesoni* and *An. rivulorum,* and 0.49 in *An. funestus s.s.* (Table 2).

6.5 kb SV insertion genotype

A total of 210 *An. funestus s.l.* samples were screened for the presence of the 6.5 kb SV insertion. Of these 38.57% (81/210) amplified and 69 were genotyped as homozygous positive (SV + /SV +), 1 as homozygous negative (SV − /SV −) and 11 as heterozygous (SV + /SV −). Overall, the frequency of the SV + allele was 0.92, with a frequency of 0.92 in *An. funestus s.s.* One unidentified mosquito was homozygous positive (SV + /SV +).

L119F-*GSTe2* mutation genotype

A total of 210 *An. funestus s.l.* samples were screened for the presence of the L119F-*GSTe2* mutation. Of these, 19.52% (41/210) amplified of which 90.24% (37/41) were homozygous susceptible and 9.76% (4/41) were heterozygous. Overall, the frequency of the L119F-*GSTe2* resistant allele was 0.05 and was only detected in *An. funestus s.s.*

Discussion

Evaluating susceptibility of local vectors to insecticides is necessary to inform the selection of appropriate vector control measures. This study evaluated the status of insecticide resistance and the potential mechanism of resistance in *An. funestus*, which is the dominant malaria vector in the Zambezia Province of Mozambique [10].

Resistance was found to all four tested insecticide classes. The highest resistance levels were observed in pyrethroid class, which is the main insecticide class used in ITNs in Mopeia. These results are in line with previous studies, which have reported rising resistance to pyrethroids in the area [10]. The increasing resistance is likely attributable to selection of pyrethroid resistant mosquitoes as a result of extended pyrethroid use in ITNs and earlier also in IRS. The partial recovery of susceptibility to pyrethroids following pre-exposure to PBO, suggests partial contribution of cytochrome *P450* monooxygenases to the resistance of *An. funestsus* to pyrethroids and that additional enzymes, such as Glutathione -S-transferases (GSTs) or additional mechanisms may be involved. Genotypic assays further confirmed the involvement of cytochrome *P450* mono-oxygenases by the presence of the 6.5 kb SV insertion gene, that is known to enhance the expression of both *CYP6P9a* and *CYP6P9b* conferring resistance to pyrethroids and carbamates. Though the frequency of the 6.5 kb SV insertion gene was high, this study shows that it is not yet fixed in Central Mozambique, unlike Southern Mozambique where fixation was reported as early as 2016. It remains to be investigated whether these differences in gene frequency indicate the spread of resistant populations from southern Mozambique or the local development of insecticide resistance.

Pirimiphos-methyl (Actellic® 300CS, Syngenta) has only been used for two years, 2016 and 2017 in Mopeia. Despite the short use, this study reports resistance to pirimiphos-methyl with the first account of resistance to pirimiphos-methyl in *An. funestsus* reported in 2019 [32]. The development of resistance to pirimiphos-methyl just 2 years after its initial use is concerning, as it suggests that other new products may also lose effectiveness quickly, given the area's history of resistance and the existing diversity of resistance and cross-resistance mechanisms in the local vector population. The reason for such rapid development of resistance could be existing selective pressure from organophosphate-based agricultural insecticides or cross-resistance with the other insecticide classes used in vector control.

The occurrence of cross-resistance between different classes of insecticides used in vector control has been recently described for pyrethroid and carbamates [33]. This cross-resistance is mediated by the cytochrome *P450 CYP6P9a* and *CYP6P9b*. The present study builds on existing evidence of resistance to carbamates in the study area. This reported resistance comes one year after the introduction and use of carbamates for IRS in the area. However, even before the application of carbamate-based IRS in the area, resistance to carbamates had already been reported further suggesting the possibility of cross-resistance or existing selection pressure from agriculture driving the carbamate resistance [10].

The current study found resistance to DDT, which contrasts with the findings of a previous 2018 assessment that reported complete susceptibility to DDT in *An. funestus s.l.* population in the area. It is possible that the resistance to DDT has developed after the previous assessment possibly as a result cross-resistance with pyrethroids due to increased pyrethroid pressure. Cross-resistance between DDT and pyrethroids is known to be conferred through mutations of insecticide binding sites such as the voltage-gated sodium chloride channels (VGSC) resulting in knock down resistance (*kdr*) or through the action of detoxification enzymes such as GSTs [34]. Genotyping assays revealed presence of L119F-*Gste2* mutation, which confers cross-resistance to DDT and pyrethroids. Albeit at a low frequency, these presence of the L119F-*Gste2* mutation reported in this study, confirms previous reports on the spread of this mutation in Eastern Africa and now in Southern Africa [35, 36].

The current study reports the presence of *Rdl* resistant alleles in the *An. funestus* population sampled. Despite this mutation being reported at high frequency in West Africa and low frequency in East Africa, to our knowledge, this study is the first report in Mozambican *An. funestus* populations [28, 37]. The persistence of the *Rdl* mutation among African malaria vectors, despite

discontinuation of dieldrin use years ago has long been puzzling [37].

Previously, the *Rdl*^{R} allele was reported to be absent in the Mozambican *An. funestus* population leading to the postulation that the *Rdl*^{R} allele is geographically restricted to West-Africa for the *An. funestus* population [28]. The current study reports the existence of *Rdl*^{R} allele in Central Mozambique. Though the use of dieldrin in Africa ceased years ago, insecticides sharing the same target site (GABA receptor), are still in use in agriculture. Fipronil, is one such insecticide that is approved and in use in Mozambique and could serve as a source of selective pressure in the mosquito population [38]. Recently, the WHO approved a new class of insecticides for vector control including broflanilide which targets the GABA receptor [39]. It would, therefore, be important to understand whether the already existing *Rdl*^{R} mutation would affect the susceptibility to broflanilide. Generally, this also highlights the need to align the agricultural sector and vector control programmes to widen the scope of insecticide resistance monitoring beyond insecticides approved for vector control, to include insecticide classes used outside of malaria vector control. Improved enforcement of regulations of insecticide use for agricultural purposes is also urgently required.

This study evaluated the presence of two metabolic resistance markers, L119F-*GSTe2* and 6.5 kb SV insertion, and one target site resistance marker, *Rdl.* While the presence of the three resistant alleles was confirmed to be present, it is worth noting that high un-amplification was observed for the metabolic markers. Previously, for the 6.5 kb insertion high rates of unamplification have been reported in some geographical regions and have been attributed to structural variation in the amplified region [36, 40]. Nevertheless, it would be important to investigate the cause of the unamplification and whether the high unamplification in the L119F-*GSTe2* mutation could also be associated with genetic variation in the region targeted by the PCR.

Conclusion

The study population of *An. funestus s.l.* from Mopeia central Mozambique was found resistant to deltamethrin, DDT, bendiocarb and pirimiphos methyl which are the major classes of insecticides used in vector control. This underscores the need to consider new insecticide classes for the insecticide-based interventions as well as non-insecticide-based tools. The high resistance to pyrethroids observed and the partial restoration of susceptibility to pyrethroids by PBO calls for consideration for the new generation of insecticide treated nets that do not rely on pyrethroids only. The occurrence of *Rdl* resistant mutation in the *An. funestus* population, despite dieldrin not being used in the area for vector control suggests a potential source of insecticide pressure is being exerted on malaria vectors perhaps from insecticides used for agricultural purposes. This highlights the need for coordinated rational insecticide usage and resistance management between health and agriculture sectors.

Abbreviations

CYP	Cytochrome P450
DDT	Dichlorodiphenyltrichloroethane
GST	Glutathione S-transferase
IRS	Indoor residual spraying
ITN	Insecticide-treated net
PBO	Piperonyl butoxide
RDL	Resistance to dieldrin locus
WHO	World Health Organization

Author contributions

Conceptualization: BC, FS, NRR, CCh and MFM. Data curation: CK and CW. Investigation: CK, LC, GC, JK, MO, and CW. Methodology: CK, CCh, and MFM. Supervision: FS and MFM. Writing—original draft: CK. Writing—review & editing: all authors contributed, reviewed and approved the last draft.

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Data availability

All data generated and analysed during this study are available in the BOHEMIA repository: https://dataverse.csuc.cat/dataverse/bohemia.

Declarations

Ethics approval and consent to participate

This study was part of a larger study evaluating the use of ivermectin for malaria control. The research protocol was reviewed and approved by the Institutional Review Board of CISM (CIBS-CISM), Research Ethics Committee of the World Health Organization (WHO-ERC), and the Research Ethics committee of University of Oxford (Oxford Tropical Research Ethics Committee). A reliance approval (based on CIBS approval) was obtained from SERU (Scientific Ethics Review Unit) of the Kenyan Medical Research Institute (KEMRI). Written informed consent was obtained from participating households prior to mosquito collections.

Competing Interests

The authors declare no competing interests.

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