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Pyrethroids and DDT tolerance of *Anopheles gambiae* s.l. from Sengerema District, an area of intensive pesticide usage in north-western Tanzania

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Abstract

OBJECTIVE To assess the susceptibility status of malaria vectors to pyrethroids and dichlorodiphenyltrichloroethane (DDT), characterise the mechanisms underlying resistance and evaluate the role of agro-chemical use in resistance selection among malaria vectors in Sengerema agro-ecosystem zone, Tanzania.

METHODS Mosquito larvae were collected from farms and reared to obtain adults. The susceptibility status of *An. gambiae* s.l. was assessed using WHO bioassay tests to permethrin, deltamethrin, lambdacyhalothrin, etofenprox, cyfluthrin and DDT. Resistant specimens were screened for knock-down resistance gene (kdr), followed by sequencing both Western and Eastern African variants. A gas chromatography–mass spectrophotometer (GC-MS) was used to determine pesticide residues in soil and sediments from mosquitoes' breeding habitats.

RESULTS *Anopheles gambiae* s.l. was resistant to all the insecticides tested. The population of *Anopheles gambiae* s.l was composed of *Anopheles arabiensis* by 91%. The East African kdr (L1014S) allele was found in 13 of 305 specimens that survived insecticide exposure, with an allele frequency from 0.9% to 50%. DDTs residues were found in soils at a concentration up to 9.90 ng/g (dry weight).

CONCLUSION The observed high resistance levels of *An. gambiae* s.l., the detection of kdr mutations and pesticide residues in mosquito breeding habitats demonstrate vector resistance mediated by pesticide usage. An integrated intervention through collaboration of agricultural, livestock and vector control units is vital.

keywords pyrethroids, resistance, agriculture, malaria vectors, Sengerema, Tanzania

Introduction

Malaria is a major threat to the survival of pregnant women and children under 5 years of age, and the great burden is in the African region south of the Sahara, where 80% of all malaria cases occur [1]. The most effective way to control malaria so far is by targeting mosquito vectors [2]. The past decade has seen a dramatic decrease in malaria infections in many malarious countries resulting from implementation of vector control interventions [3, 4]. However, vector control measures such as indoor residual spraying (IRS), insecticide-treated nets (ITNs) and recently the use of long-lasting insecticidal nets (LLINs) depend on the susceptibility of mosquitoes to insecticides. Unfortunately, the current vector control interventions are severely threatened by the emergence and spread of insecticide resistance among malaria vectors [5]. Indeed, in some of the resistance reports, ITNs and IRS use have been implicated as sources of selection pressure [6, 7]. Nonetheless, several factors may have contributed to vector resistance, including the use of the same chemical classes of insecticides in agriculture [8–10] and the public health sector [11].

Currently, most vector control programmes are largely dependent on synthetic pyrethroids such as deltamethrin, permethrin, lambdacyhalothrin, cyfluthrin and etofenprox, which are the only ones recommended by the WHO for insecticide-treated net (ITN) treatment and widely used in IRS programmes. This is due to their relatively low mammalian toxicity and rapid knock-down effect that causes paralysis of an insect [12]. Our Knowledge, Attitude and Practice (KAP) Survey (our unpublished findings) revealed that the same pyrethroids (deltamethrin, permethrin and lambdacyhalothrin) are

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also used in agriculture for control of both crop and livestock pests and this intensive dual use has led to reduced efficacy [13–15]. Agricultural fields form important breeding habitats for disease vectors; hence, selection pressure increases due to multiple exposures to insecticides. The use of pyrethroid insecticides for malaria vector control in Tanzania has risen dramatically in the past decade through the scale up of universal distribution of ITNs and IRS campaigns [6]. Although the use of pyrethroids for the control of crop pests is also on the increase in Tanzania [16, 17], the role of these pesticide residues in mosquito breeding habitats on the development of vector resistances has been hypothesised without clear evidence [10, 18, 19]. This study, therefore, is a step towards understanding the interaction between malaria vectors, breeding habitats and pesticide residues and their contribution to the development of insecticide resistance in malaria vectors.

Several studies have shown increasing trends of vector resistance to pyrethroids and DDT, as well as the knockdown resistance (kdr) mutations in the anopheline voltage-gated sodium channel (VGSC) gene [7, 19, 20]. Insect mechanisms in response to insecticides basically involve two ways: either metabolic detoxification of the insecticide before it reaches its target site, or changes in sensitivity of the target site so that it is no longer susceptible to insecticide inhibition [21]. The metabolic-based mechanisms of resistance have been reported in populations of *An. arabiensis* from north-west Tanzania [18]. Similarly, the kdr mutation involving the substitution of leucine to serine at amino acid position 1014 (L1014S) of the VGSC gene (East African kdr) has been widely reported in *An. gambiae* s.l. populations from various parts of the country [22, 23]. The West African kdr mutation L1014F has also been detected in the population of *An. gambiae* s.l. from different places in the country [20, 24]. In all cases, the mutated alleles conferring resistance in malaria vectors have been ascribed to increased use of insecticides for public health in form of ITNs and IRS [6, 20] in the country.

Nevertheless, Nkya *et al.* [19] suggested the possibility of developing multiple mechanisms of resistance in an individual insect as a result of multiple exposures of disease vectors to agricultural pollutants. The use of pesticide cocktails in agricultural fields where disease vectors also breed further serves to complicate issues such as development of vector resistance and multiple resistance mechanisms in insect vectors (personal observation). The application of pesticide cocktails against crop pests has been reported by other scholars [25, 26]. Varieties of pesticides are constantly used against crop pests and in the form of cocktails in Tanzania [17, 27, 28], also abusively as in overuse and overdose. As the same agricultural areas are important breeding habitats for malaria vectors [29, 30], pesticide and/or insecticide resistance among disease vectors is an overlapping issue between the fields of agriculture and public health. However, the relationship between pesticide usage and development of vector resistance in their natural habitats has not been well established in the country. Thus, the extent of selection pressure of disease vectors resulting from pesticide usage in agricultural fields may be underestimated.

In the light of current knowledge of misuse of pesticides in malaria-endemic regions, it was the aim of this study to assess the role of agricultural pesticides in the development of resistance to public health insecticides in malaria vectors from the Sengerema agro-ecosystem zone of Tanzania. Our findings are important to policymakers overseeing the vector and pest control units for evidence-based decision-making.

Materials and methods

Study site

The study was conducted in Sengerema District, Mwanza region, of Tanzania (Fig. 1). Malaria is endemic in Sengerema District with the estimated prevalence of >40% for children between 2 and 10 years of age [31]. In Tanzania, particularly in the study area, the use of pesticides and fertilisers dates back to the colonial era, when they were introduced to control pests of important cash crops and to improve soil fertility [16]. In the study area, pesticides are mainly applied to cotton and horticultural crops, mostly in the form of fungicides, insecticides and herbicides [32]. Furthermore, there is a history of DDT use in the area, but that was banned in the 1990s due to its detrimental effects on the environment [33].

Currently, pesticides that are commonly used for both cotton and horticultural fields in this area are the pyrethroids (cypermethrin, deltamethrin, cyfluthrin, cyhalothrin and fenvalerate); organophosphorus and carbamates are used to a lesser extent [33].

Mosquito larvae were sampled between January and September 2014, covering the main cropping seasons of the year (long rain, dry and short rain seasons). The majority of residents in this district are engaged in small-scale farming; the cash crops grown are cotton and vegetables such as tomatoes and cabbages. Mixtures of pesticides were intensively applied, particularly on cotton and tomato farms, which are also breeding areas for mosquitoes. The choice of the study sites therefore considered malaria endemicity, past and current use of pesticides in agriculture and public health and availability of mosquito

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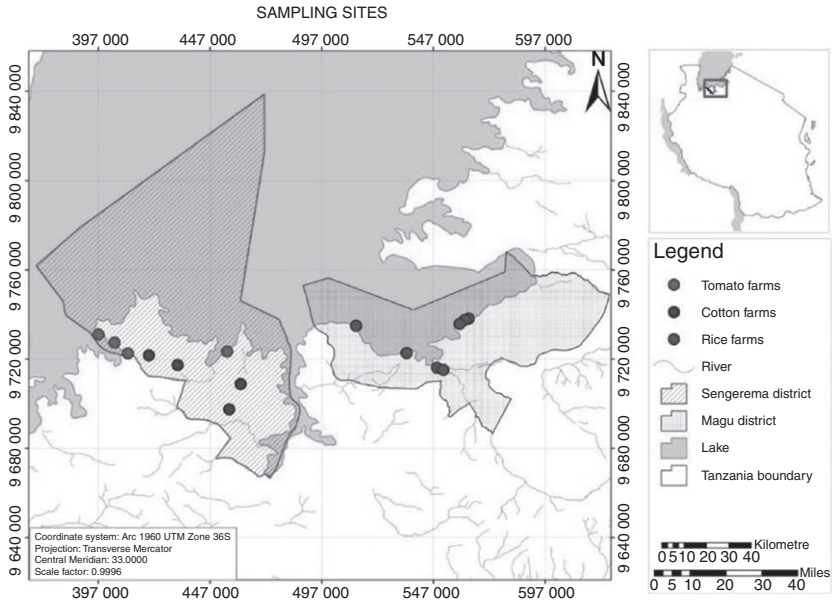


Figure 1 Map of Tanzania showing location of the study sites (Source: GIS center, Serengeti National Park).

breeding habitats. Tomatoes were cultivated at a large scale and under irrigation, which ensured availability of mosquito breeding habitats throughout the year.

Larvae sampling and rearing

Larvae and pupae were collected from cotton and tomato farms and brought to the insectarium at the National Institute for Medical Research (NIMR), Mwanza Center, for rearing to obtain adults. The collected larvae were fed on tetramine fish food and reared at laboratory conditions of 25–27 °C and 67–79% relative humidity [34]. On pupation, pupae were transferred to rearing cages, the emerging female adults were morphologically identified using published keys [35, 36], and only members of *An. gambiae* s.l. were used for susceptibility testing.

Susceptibility test

The susceptibility test followed the WHO guidelines [37]; WHO test kits and control papers were supplied by the WHO Collaborating Centre at Universiti Sains Malaysia, Penang, Malaysia. The insecticides tested were the five pyrethroid compounds commonly used for both agriculture and vector control, that is permethrin (0.75%), deltamethrin (0.05%), lambdacyhalothrin (0.05%), etofenprox (0.5%) and cyfluthrin (0.15%), and the organochlorine DDT (4%). *Anopheles gambiae* s.l. mosquitoes were exposed to the insecticides for one hour; the number of knock-down mosquitoes was recorded at the time intervals (10, 15, 20, 30, 45, 60 min). A laboratory-susceptible strain of *An. gambiae* Kisumu was used as a reference to compare the susceptibility levels of the field populations; mortalities were scored after a 24-h holding period [37], during which the mosquitoes were consistently fed on sugar solution. According to the WHO (2013) guidelines, when the mortality rate is >98%, the population is considered fully susceptible; a mortality rate of 97–98% means resistance is suspected, and when the mortality rate is <97%, it means the population is resistant and resistance genes have to be confirmed.

Sibling species identification, kdr genotyping and sequencing

Genomic DNA was extracted from individual mosquitoes following the procedure of Collins *et al.* [38] and stored at −20 °C till use for polymerase chain reactions (PCR). Identification of *An. gambiae* sibling species was performed by PCR according to Scott *et al.* [39] using the reverse primers CTGGTTTGGTCGGCACGTTT for *An. gambiae* s.s., AAGTGTCCTTCTCCATCCTA for *An. arabiensis*, TGACCAACCCACTCCCTTGA for *An. Merus* and CAGACCAAGATGGTTAGTAT for *An. Quadriannulatus,* and the species-independent universal forward primer 5′ GTGTGCCCCTTCCTCGATGT 3′ [39]. Detection of the West African L1014F and East African L1014S kdr alleles involved the allele-specific AS-

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PCR adapted from Martinez-Torres *et al.* [40] and Ranson *et al.* [41], respectively. To further confirm the mutations, the PCR products from the amplified kdr gene from kdr-positive mosquitoes were ligated into the pGEM-T Easy vector (Promega) and transformed into *E. coli* NovaBlue cells (Novagen) as described by Ngwa *et al.* [42]. Colony PCR followed by agarose gel electrophoresis was used to verify the kdr gene using the T7 (GTAATACGACTCACTATAGGG) and SP6 (ATT TAGGTGACACTATAG) pGEM-T Easy vector primers to flank the mutant gene. One positive clone was selected, and plasmid DNA was isolated using the NucleoSpin plasmid extraction kit (Macherey-Nagel). The kdr gene was then sequenced using the T7 primers. Sequencing was performed in the Fraunhofer Institute for Molecular Biology and Applied Ecology Aachen, Germany. The specific kdr gene mutation was then determined by sequence analyses.

Sampling of soils and sediments for pesticide residue determination

A total of 24 soil and sediment samples were collected from sampling points in the cotton and tomato farms in the studied area. Sediments were collected under water in a particular mosquito breeding habitat/microhabitat, whereas the soils were sampled at the depth of 20 cm in various sampling points in each farm. Both sediments and soils were simultaneously collected from the mosquito breeding habitats in the particular farm at the same time when mosquito larvae were collected. Samples were wrapped in aluminium foil, labelled and deep-frozen at −20 °C immediately after sampling and prior to transportation to the laboratory for analysis.

Pesticide residue extraction and analysis

Extraction and clean-up of the compounds were performed by the solid dispersion and Florisil method following the detailed procedures outlined in the Environmental Monitoring Guidelines for SADC Region [43]. The concentration of pesticide residues was determined using a gas chromatography–mass spectrophotometer (GC-MS), model QP2010 ultra, equipped with flame ionisation detector (FID). The injection was performed using 2 µl of 5% phenyl polysiloxane was injected into the GC-MS at 310 kpa (pulse pressure). The injector and ionisation temperatures were set at 208 °C and 230 °C, respectively. Helium flowing at a rate of 1 ml/min was used as a carrier gas. Column temperature was set at 70 °C/2 min at a rate of 25 °C/min, then increased to 150 °C for 3 min and 200 °C for 8 min and finally elevated to 280 °C and maintained at this temperature for 10 min. The GC-MS analyses followed the published procedure as narrated by Brunete *et al.* [44]. The extraction of pesticide residues and analysis were performed at the Department of Chemistry, University of Dar es salaam, Tanzania. All reagents used were of analytical grade, and the solvents were of chromatographic grade at 99% purity.

Data analysis

The time taken for 50% and 95% of all mosquitoes to knock down (knock-down times) was estimated by the log probit model analysis using the time–mortality curves. The software used was Origin Pro 8.5, version 84E, 2010, Origin Lab Corporation. The resistance ratios (RR) were calculated using the KDT50 between the wild mosquitoes and the susceptible laboratory strain (Kisumu). The differences in the susceptibility of *An. gambiae* s.l. to various insecticides, from different farms and different seasons, were computed by two-way ANOVA followed by multivariate analysis. The software used is GraphPad version 6.01. The genotype and allele frequencies were computed using the Hardy–Weinberg equation. The concentration and identification of pesticide residue compounds were determined using reference standards (commercial), relative retention times and mass-to-charge ratios (GS-MS, model QP2010).

Results

Sibling species identification and susceptibility test results

A total of 3380 adult mosquitoes obtained from larvae rearing were morphologically identified as *An. gambiae* s.l. and were exposed to various insecticides (220–300 mosquitoes per insecticide for the three seasons). A total of 1066 *An. gambiae* s.l. mosquitoes (701 mosquitoes randomly withdrawn from the samples that died after insecticide exposures and 305 mosquitoes that survived the exposures) were genotyped for sibling species identification. Of these, 2% were *An. gambiae* s.s., 91% were *An. arabiensis*, and 6.7% could not be identified.

Mortality rates

Analysis of the data shows that seasonality did not influence mortality rates of *Anopheles gambiae* s.l. collected from the cotton farms following exposure to insecticides (permethrin, deltamethrin, lambdacyhalothrin, etofenprox and cyfluthrin and DDT – Table 1) ($P = 0.437$, $F_{(2,54)} = 0.84$). However, the mortality rate significantly

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varied between individual insecticides, as shown by two-way ANOVA and Turkey post hoc test ($P < 0.0001$, $F_{(5,54)} = 8.58$). On the contrary, the mortality rate of *An. gambiae* s.l. collected from the tomato farms significantly varied with seasons ($P = 0.0002$, $F_{(2,54)} = 10.03$). Similarly, mortality rate significantly varied between individual insecticides, as demonstrated by two-way ANOVA followed Turkey post hoc test ($P = 0.026$, $F_{(5,54)}$).

Multiple comparison of all variables between the two farms (cotton and tomato) revealed that mortality rates of *An. gambiae* s.l. significantly varied with season ($P < 0.0001$, $F_{(5,36)} = 11.38$) and with particular insecticides ($P < 0.0001$, $F_{(1,36)}$).

In all control groups, the mortality rate was consistently <5% throughout the experimental time; thus, the correction factor for the control mortality was not applied. Furthermore, the reference strain (Kisumu) was fully susceptible (100% mortality) after exposure to all the pyrethroids and DDT tested.

Knock-down times

Resistance to pyrethroids and DDT was associated with increased knock-down times, which were measured between 10 and 60 min of exposure (Table 2). The knock-down time (KdT_{50}) was up to 182 min for lambdacyhalothrin. Similarly, the resistance ratio (RR) was generally high, up to 6.4, suggesting a kdr-related mechanism of resistance. However, there was no significant difference for the time taken to knock down 50% of the mosquitoes (KdT_{50}) for all insecticides tested between the two farms ($t = 0.428$, $P = 0.675$) by paired-sample t-test. Resistance ratios for all insecticides generally did not significantly differ between the two farms in all seasons, $P > 0.05$.

Knock-down resistance mutations (kdr)

The 305 *An. gambiae* s.l. mosquitoes that survived DDT and pyrethroid exposures were genotyped for kdr mutations. Diagnostic PCR revealed that only 13 specimens were positive for the kdr Eastern variant (L1014S). The Western African kdr mutation (L1014F) was not detected in any of the specimen. In general, the susceptible wild-type genotype was the most prevalent with 95.8% abundance; the heterozygous and homozygous kdr resistance genotypes were prevalent with 2.6% and 1.2% abundance, respectively (Fig. 2, Table 3). The allelic frequencies of kdr and Eastern variant (L1014S) from both farm types and across the seasons were in the range of 0.9–50%.

Pesticide residues

The pesticide residues of the soil and sediment samples that were collected from mosquito breeding habitats were identified via GC-MS. Only DDT and its metabolites (DDD DDE such as *o, p'*-DDD; *p, p'*-DDD; *p, p'*-DDE; *p, p'*-DDT) were detected above the method detection limits (MDLs) in all soil and sediment samples (Table 4). The concentrations of the residues were generally low and ranged from trace–9.90 ng/g (dry weight) and from trace–2.04 ng/g for the soils and sediment samples, respectively. The total concentrations of pesticide residues in the soil and sediment samples were 20.32 ng/g (dry weight) and 5.65 ng/g (dry weight), respectively.

Table 1 Mortality rates of *An. gambiae* s.l. 24 h post-exposure to pyrethroids and DDT in the WHO test kits, January-September, 2014

Type of Farm	Insecticide tested	% Mortality Long rains season ± SD	% Mortality Dry season ± SD	% Mortality Short rains season ± SD
	Kisumu	100 ± 0	100 ± 0	100 ± 0
Tomato	Permethrin 0.75%	73 ± 5.715	87.5 ± 8.66	100 ± 0
	Deltamethrin 0.05%	88.25 ± 9.94	96.25 ± 4.9	91.5 ± 12.77
	Lambdacyhalothrin 0.05%	63 ± 20	85 ± 9.13	93.25 ± 9.4
	Etofenprox 0.5%	84.75 ± 6.18	86.75 ± 17.5	98.25 ± 3.5
	Cyfluthrin 0.15%	85.75 ± 16.17	90.75 ± 4.2	98.25 ± 3.5
	DDT 4%	90 ± 20	90.75 ± 7.28	100 ± 0
Cotton	Permethrin 0.75%	81 ± 13.58	75 ± 21.2	73 ± 5.71
	Deltamethrin 0.05%	76.25 ± 8.65	80 ± 12.9	86.25 ± 5.3
	Lambdacyhalothrin 0.05%	79.75 ± 23.93	86 ± 11.34	59.5 ± 13
	Etofenprox 0.5%	79.5 ± 12.23	98.25 ± 3.5	94.75 ± 6.7
	Cyfluthrin 0.15%	96.5 ± 4.04	89.75 ± 11.64	89.5 ± 7
	DDT 4%	96.5 ± 4.04	98.25 ± 3.5	100 ± 0

SD is standard deviation: Kisumu is the susceptible laboratory strain used for reference.

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Table 2 Knock-down times (KdT50 and KdT95) in minutes and resistance ratios (RR) for *Anopheles gambiae* s.l. from cotton and tomato farms in Sengerema District, north-western Tanzania

Season	Farm	Insecticide	KdT_{50} Kisumu	KdT_{50} (*An. gambiae* s.l.)	KdT_{95} (*An. gambiae* s.l.)	$KdT_{50}R$
Long rains	Cotton	Permethrin 0.75%	44.74 [41–46]	47.59 [42–58]	75.41 [64–95]	1.06
		Deltamethrin 0.05%	20.38 [17–22]	39.20 [37–41]	61.83 [59–66]	1.92
		Lambdacyhalothrin 0.05%	28.56 [28–30]	76.20 [44–150]	129.64 [69–273]	2.67
		Etofenprox 0.5%	27.00 [25–28]	44.87 [39–55]	69.42 [60–88]	1.66
		Cyfluthrin 0.15%	16.23 [13–20]	37.40 [36–42]	58.87 [56–83]	2.3
		DDT 4%	35.14 [34–36]	38.81 [37–41]	58.99 [56–64]	1.1
	Tomato	Permethrin 0.75%	44.74 [41–46]	46.31 [36–58]	75.66 [65–95]	1.03
		Deltamethrin 0.05%	20.38 [17–22]	36.12 [33–38]	59.24 [56 –62]	1.77
		Lambdacyhalothrin 0.05%	28.56 [28–29]	97.52 [59–14]	169.46 [97–252]	3.41
		Etofenprox 0.5%	27.00 [25–28]	35.58 [34–37]	55.22 [53–57]	1.32
		Cyfluthrin 0.15%	16.23 [13–20]	46.33 [42–48]	72.95 [65–76]	2.85
		DDT 4%	35.14 [34–36]	46.33 [42–48]	72.95 [65–76]	1.32
Dry	Cotton	Permethrin 0.75%	44.74 [41–46]	57.10 [28–86]	93.57 [70–130]	1.27
		Deltamethrin 0.05%	20.38 [17–22]	38.18 [34–50]	67.26 [58–85]	1.87
		Lambdacyhalothrin 0.05%	28.56 [28–30]	47.93 [44–51]	80.29 [70–87]	1.69
		Etofenprox 0.5%	27.00 [25–28]	37.81 [37–39]	58.43 [56–61]	1.4
		Cyfluthrin 0.15%	16.23 [13–20]	38.41 [36–24]	61.36 [58–67]	2.37
		DDT 4%	35.14 [34–36]	39.51 [37–40]	61.91 [59–67]	1.12
	Tomato	Permethrin 0.75%	44.74 [41–46]	44.89 [38–54]	70.72 [60–86]	1
		Deltamethrin 0.05%	20.38 [17–22]	34.97 [34–37]	56.29 [54–60]	1.72
		Lambdacyhalothrin 0.05%	28.56 [28–29]	75.77 [53–103]	127.56 [84–180]	2.65
		Etofenprox 0.5%	27.00 [25–28]	47.23 [39–53]	75.31 [61–88]	1.75
		Cyfluthrin 0.15%	16.23 [13–20]	32.71 [22–40]	59.24 [53–68]	2.02
		DDT 4%	35.14 [34–36]	44.84 [38–47]	71.05 [59–76]	1.28
Short rains	Cotton	Permethrin 0.75%	44.74 [41–46]	47.69 [40–54]	73.80 [60–85]	1.06
		Deltamethrin 0.05%	20.38 [17–22]	35.53 [32–38]	61.33 [57–65]	1.74
		Lambdacyhalothrin 0.05%	28.56 [28–30]	182.83 [97–362]	330.19 [167–671]	6.4
		Etofenprox 0.5%	27.00 [25–28]	43.76 [37–47]	67.54 [58–73]	1.62
		Cyfluthrin 0.15%	16.23 [13–20]	34.55 [32–37]	60.55 [59–63]	2.13
		DDT 4%	35.14 [34–36]	45.69 [44–49]	70.71 [68–77]	1.3
	Tomato	Permethrin 0.75%	44.74 [41–46]	44.31 [36–67]	70.50 [55–110]	0.99
		Deltamethrin 0.05%	20.38 [17–22]	41.55 [29–51]	69.91 [52–85]	2.04
		Lambdacyhalothrin 0.05%	28.56 [28–29]	67.59 [54–82]	112.28 [91–140]	2.36
		Etofenprox 0.5%	27.00 [25–28]	39.59 [39–40]	61.96 [61–65]	1.46
		Cyfluthrin 0.15%	16.23 [13–20]	49.27 [41–61]	79.66 [65–102]	3.04
		DDT 4%	35.14 [34–36]	56.20 [40–101]	90.95 [61–174]	1.59

KdT50 refers to time taken for 50% of tested mosquitoes to knock down. $KdT_{50}R$ is the resistance ratio (RR) obtained by dividing the KdT_{50} of the tested wild population by the KdT_{50} of the 'Kisumu' susceptible strain.

Discussion

The present study investigated the role of agricultural pesticide in the development of resistance in malaria vectors in Sengerema District, which is the area of intensive pesticide usage in northern Tanzania. Our data demonstrate that the population of malaria vectors in the studied area is dominated by *An. arabiensis*. Several studies have reported the predominance of *An. arabiensis* over other sibling species in the *Anopheles gambiae* complex [45-48]. This behaviour has been associated with the development of adaptive traits as a result of long-term use of ITNs that increased the proportion of *An. arabiensis* (exophagic species) as opposed to *An. gambiae s.s.*, which were mainly targeted by insecticides inside houses. The predominance of *An. arabiensis* over the other sibling species is of paramount importance because of the existing vector control programmes that target vectors within the house (ITNs and IRS). Therefore, the current interventions IRS and ITNs need to be complimented by additional methods that will effectively control *An. arabiensis*, which now dominates the *Anopheles gambiae* species complex.

The susceptibility of *An. gambiae* s.l. to insecticides varied with seasons; during the long rain season, high

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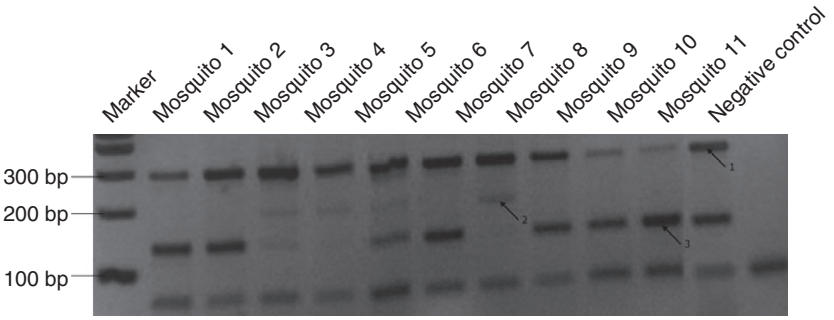


Figure 2 Genotyping of kdr East mutation by PCR-based diagnostic test. The figure shows an extract of the genotyping results. A diagnostic PCR was carried out using DNA extracted from each mosquito followed by separation of the PCR production on a 2% agarose gel (11 mosquitoes shown). Band 1 shows the internal control band (293 bp) which is present in all mosquitoes tested. Band 2 shows the susceptible band (137 bp) and band 3 (195 bp) the kdr resistant band. Mosquitoes 1, 2, 6, 8, 7, 9, 10, 11 are susceptible genotype (SS); mosquitoes 4 and 7 are hetrozygous resistant (RS); mosquitoes 3 and 5 are homozygous resistant (RR).

Table 3 Genotype and allele frequencies (kdr L1014S) in populations of *Anopheles gambiae* s.l. according to their survival in the bioassay test (all the seasons pulled together)

Type of farm	Insecticide tested	No tested	No of survivors	Genotypes			Genotype frequency			Allele frequency	
				RR	RS	SS	RR	RS	SS	R	S
Cotton	Permethrin 0.75%	300	62	1 (arab.)	0	61	0.016	0	0.983	0.016	0.983
	Deltamethrin 0.05%	300	31	0	0	31	0	0	1	0	1
	Lambdacyhalothrin 0.05%	290	56	0	1 (s.s)	55	0	0.018	0.982	0.009	0.991
	Etofenprox 0.5%	270	14	0	1 (s.s)	14	0	0	1	0	1
	Cyfluthrin 0.15%	270	28	0	0	28	0	0	1	0	1
	DDT 4%	220	3	1 (s.s)	1 (s.s)	1	0.333	0.333	0.333	0.5	0.5
Tomato	Permethrin 0.75%	300	22	0	0	22	0	0	1	0	1
	Deltamethrin 0.05%	300	22	2 (s.s)	2 (s.s)	18	0.091	0.091	0.818	0.136	0.864
	Lambdacyhalothrin 0.05%	270	27	0	1 (arab)	26	0	0.037	0.962	0.018	0.981
	Etofenprox 0.5%	300	22	1 (arab.)	2 (arab)	18	0.045	0.136	0.818	0.113	0.886
	Cyfluthrin 0.15%	260	10	0	0	10	0	0	1	0	1
	DDT 4%	300	8	0	0	8	0	0	1	0	1
Total		5160	435	5	8	422	0.009	0.018	0.97	0.018	0.982

R = resistant L1014S allele; S = susceptible wild type; RR = homozygous resistant; RS = heterozygous; SS = homozygous wild type: arab = *An. arabiensis* and s.s = *An. gambiae* s.s.

levels of resistance to all insecticides were observed. The mortality rate for the tomato farm samples was as low as 63% and 73% after exposure to lambdacyhalothrin and permethrin, respectively. This was not a surprise as pyrethroids are extensively applied in tomato farms. In addition, the long rain season coincided with pesticide applications in farms, and during larvae sampling, many farmers were seen spraying tomatoes and other vegetables almost throughout the sampling season (personal observations). Heavy rainfall was also common during this time, which could have facilitated run-offs, and the pesticide residues could easily have been deposited in the adjacent mosquito breeding habitats, thereby increasing selection pressure. The high resistance level against lambdacyhalothrin was matched with increased knock-down times (KDT50) and resistance ratios. The knock-down time of this population when exposed to lambdacyhalothrin was 97.20 min during the season of long rains and up to 182 min during the season of short rains. The resistance ratio was >2, suggesting kdr-based mechanisms of resistance. Amino *et al.* [49], using quantitative imaging of *Plasmodium* transmission from mosquito to a mammal, showed that it was able to transmit malaria parasites within 1 min. Other studies have shown that

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Table 4 Concentration of pesticide residues (ng/g/dry weight) in soils and sediment samples from the mosquito breeding habitats in the cotton and tomato farms of Sengerema agro-ecosystem zone, north-western Tanzania

			Pesticide standards										
Type of Farm	Number of samples	*o,p'*-DDD	*p,p'*-DDD	*o,p'*-DDE	*p,p'*-DDE	*o,p'*-DDT	*p,p'*-DDT	Deltamethrin	α-Endosulfan	β-Endosulfan	Fenitrothion	Pirimiphos-methyl	Cypermethrin
Tomato	Soils (6)	1.50	9.90	nd	nd	nd	5.82	nd	nd	nd	nd	nd	nd
	Sediments (6)	nd	1.08	nd	1.03	nd	nd	nd	nd	nd	nd	nd	nd
Cotton	Soils (6)	nd	3.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Sediments (6)	3.54	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

nd = not detected; conc. in ng/g = μg/kg (ppb); *N* = sample size.

the average time for malaria parasite transmission from a mosquito to a mammal is 3 min [50], with a large number of sporozoites being released within the first and second salivation for up to 10 min of mosquito probing [51]. Therefore, if the insecticides currently in use may take up to 182 min to knock down 50% of the mosquitoes (Table 2), it is possible that mosquitoes will knock down after they have transmitted malaria parasites.

During the period of short rains, *An. gambiae* s.l. mosquitoes from tomato farms were highly susceptible to all insecticides tested. The possible explanation for this is that tomato plants were being replaced, and in some farms, the plants were very young and no pesticides were applied during that time. Given the high degradation rate of pyrethroids [52], short-time residues' persistence in the breeding habitats may have rendered the vectors fully susceptible. By contrast, *An. gambiae* s.l. from the cotton farms was resistant to permethrin, deltamethrin and lambdacyhalothrin. The high resistance levels of mosquito populations were coupled with increased knockdown times and high resistance ratios. There was no pesticide usage during the short rain season as the cotton plants were fully grown. Considering the fact that vector resistance is also an evolutionary phenomenon [53], it is possible that mosquitoes had acquired resistance genes from their parents due to pesticide usage in the previous seasons; therefore, the genes are being passed from one generation to another.

Molecular analysis of the resistant specimens revealed the leucine–serine (L1014S) mutation in the sodium channel gene of both *An. gambiae* s.s. and *An. arabiensis*. The occurrence of this mutation in both species of *An. gambiae* s.l. complex may mean that the species have similar exposure to sources of insecticides causing the knockdown selection pressure. The ongoing active agricultural activities involving use of pesticides are likely a source of selection pressure for reduced sensitivity to pyrethroids in the *An. gambiae s.l.* population and hence the knockdown mutations. The kdr mutations, however, were more frequently found in *An. gambiae* s.s. than in *An. arabiensis* (Table 3). Less frequent occurrence of the kdr allele in the *An. arabiensis* population than in *An. gambiae* s.s. has also been reported from Burkina Faso [54]. The difference in the frequency of kdr mutation in the two species may mean that *An. gambiae* s.s. is more susceptible to the kdr mutations than *An. arabiensis*. This could have resulted due to frequency exposure of insecticides in the form of ITNs and IRS for many years to *An. gambiae* s.s. This species feeds and rests indoors as opposed to *An. Arabiensis,* which is exophagic. However, the operational significance of these observations is uncertain given the high levels of phenotypic resistance observed in the

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population of *An. gambiae* s.l. in which *An. arabiensis* constitutes over 90% of the total population.

Despite the high levels of phenotypic resistance in the population of *An. gambiae* s.l. against permethrin and lambdacyhalothrin recorded during insecticide exposures, the kdr mutations (L1014S) were only detected in one and two specimens that, respectively, survived permethrin and lambdacyhalothrin exposures. This observed low kdr mutation frequency to pyrethroids is consistent with previous studies [54, 55], in which kdr mutations were closely associated with resistance to DDT and weakly associated with resistance to pyrethroids. These findings suggest that other resistance mechanisms against pyrethroids may be active in the population of *An. gambiae* s.l. from this area, possibly attributable to pesticide cocktails applied by farmers in the area studied. Similarly, although phenotypic resistance was also recorded in the same population against cyfluthrin, no mutations were detected in any specimen that survived cyfluthrin exposure, suggesting that the observed resistance could be solely attributed to metabolic mechanisms whose detection was out of the scope of this study.

It was also observed that farmers applied mixtures of pesticides (herbicides, insecticides, fungicides, etc.), and the pesticide cocktails were also made up of different chemical groups (Our unpublished KAP Survey data). Thus, it is not surprising that *An. gambiae* s.l. mosquitoes have been selected for different resistance mechanisms to survive multiple exposures of insecticides that are responsible for high phenotypic resistance levels recorded, and these mechanisms could not be detected by the conventional methods employed in the present study. This discrepancy, however, requires further investigations.

The analysis of pesticide residues only detected DDT and its metabolites (DDE and DDD) in the soils and sediment samples collected from the mosquito breeding habitats. The presence of DDTs in this area can be related to past use of the pesticide in agricultural fields. Substantial amounts of DDT residues have also been reported from other parts of the country [56, 57], approximately 20 years after its official ban for agricultural use in Tanzania. Although pyrethroids are widely used in the area (unpublished observations) against crop and livestock pests, none of the pyrethroids was detected during the pesticide residue analysis. The plausible explanation for this is because pyrethroids undergo rapid degradation [52], do not persist in the soil and cannot, therefore, be easily detected in the environment. However, the presence of DDT and its metabolites in the mosquito breeding habitats is of great concern in the management of disease vectors. DDT shares a similar mode of action with pyrethroids. Pyrethroids are the only class of insecticide recommended for treatment of nets (ITNs) and widely used in IRS programmes against mosquito vectors. Cross-resistance of DDT/pyrethroids has been reported from several insect species [58–60] and also in disease vectors [40, 61]. Therefore, the observed high levels of resistance against pyrethroids in the population of *An. gambiae* s.l. from the study area might also be due to cross-resistance from DDT residues present in the mosquitoes' breeding habitats.

Conclusion

This study reports the predominance of *An. arabiensis* over other sibling species of the wild *An. gambiae* s.l. complex population, which calls for modified vector control interventions targeting this species. The recorded high resistance levels and knock-down times, together with high resistance ratios of *An. gambiae* s.l. to pyrethroids and DDT, imply reduced insecticide efficacies. The detection of DDT residues in the mosquitoes' breeding habitats calls for adoption of integrated pest management that will reduce the use of pesticides and hence reduction of multiple exposures of pests and disease vectors to pesticides. This can be achieved through collaboration of relevant sectors such as agriculture, livestock and vector control units, which are hereby encouraged to consider joint ventures for any intervention.

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