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Multiple Pyrethroid Resistance in Urban Male and Female *An. gambiae s.l* Populations in Ilorin Nigeria: Implications for Swarm Spraying and Toxic Sugar Bait Malaria Vector Control

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ABSTRACT

Background: Male mosquito control through swarm spraying and Attractive Toxic Sugar Baits (ATSB) have been identified as potential strategies to complement the regular but insufficient female mosquito-based insecticide-treated net (ITN) approach. However, the effectiveness of these control strategies relies on understanding the insecticide susceptibility status of the targeted male and female mosquito populations. Therefore, this study aimed to assess the insecticide susceptibility status of male and female *An. gambiae s.l.* Populations in urban Ilorin, Nigeria.

Methods: Using WHO kits and protocol, adult male and female mosquitoes reared from the same larval collections from different sites were exposed separately to permethrin, deltamethrin, and alphacypermethrin insecticides. Joint insecticide susceptibility tests, involving exposures of equal numbers of male and female mosquitoes to each insecticide, were also conducted in anticipation of joint insecticide exposure on the field. Exposed mosquitoes were identified to species level.

Results: Higher kdt_{50}s (52.5-641.3mins) and lower mortalities (permethrin 39-62%, deltamethrin 57-68%, alphacypermethrin 44-60%), indicating higher levels of resistance, were observed for the females compared to the male (22.1-42.2 mins, 60-94%) mosquitoes. The percentage mortalities of males (71-94%) were significantly ($P \leq 0.043$) higher than those of the jointly exposed females (39-62%). However, separately exposed male mosquito mortalities (60-81%) were not significantly ($P \geq 0.067$) higher than the females (55-68%). All the male mosquito populations were resistant to permethrin (75-94% mortality), deltamethrin (60-82%), and alphacypermethrin (74-86%) insecticides after joint and separate exposures. Resistant male and female mosquitoes were identified predominantly as *An. arabiensis* (74-84%).

Conclusion: Multiple pyrethroid insecticide resistance detected in the male and female mosquito populations indicates the potential ineffectiveness of pyrethroid-based swarm spraying, pyrethroid ATSB and ITNs in the study location

Keywords: *Anopheles*, Male mosquito, Insecticide resistance, Swarm control, Malaria

1.0 INTRODUCTION

Global malaria burden declines have been reported progressively over several years of increased malaria vector control interventions using insecticide-treated nets (ITN) and indoor residual spraying (IRS) of recommended insecticides. Globally, over 2.3 billion ITNs (86% to sub-Saharan Africa (SSA)) were delivered by ITN manufacturers between 2004 and 2020 [1]. Between 2000 and 2015, a significant 40% reduction in clinical malaria incidence was reported, with widespread insecticide-treated net (ITN) interventions contributing to 68% of the 663 million clinical malaria cases averted in endemic Africa [2]. Concurrently, 68% (50/73) of malaria-endemic countries reporting insecticide resistance (IR) data for 2010-2016 recorded female malaria mosquito resistance to two or more insecticide classes [3]. In 72 malaria-endemic countries, pyrethroid insecticide resistance frequency increased from 26% to 58% in female *An. funestus s.l* and from 21% to 34% in female *An. gambiae* s.l. between 2010 and 2016 [4]. The widespread reports of female mosquito IR threaten malaria vector control and sustainable reduction in disease incidence, especially in sub-Saharan Africa. Impressive reductions in global malaria incidence (41%) and mortality rates (62%) were observed between 2000 and 2015 [3]. However, progress has stalled since 2015, with minimal changes in both rates between 2015 and 2018 [5]. Complementary intervention strategies are required to control vector species and populations not effectively targeted by current ITN and IRS tools to sustain malaria vector control gains recorded [6]. For instance, the male mosquito populations contribute significantly to reproduction and increasing population density of the female malaria vectors [7]. Male mosquito interventions such as the use of attractive toxic sugar baits (ATSB) [8,9] and spraying of swarms with insecticides [6] could decrease male mosquito density thereby reducing female mating and overall mosquito vector population density. Both male and female mosquitoes use plant sugars as an energy source [9], and those lacking adequate access to blood often satisfy their increased energetic requirements by consuming more sugar [10]. Accordingly, suitable insecticides used successfully against mosquitoes in outdoor ATSB [8,11] could be deployed against male and host-seeking female mosquitoes indoors through indoor ATSB combining concentrated sugar-based food source, an olfaction stimulant, and an oral insecticide to lure and kill mosquitoes [9]. In addition, evidence of swarm disruption towards a mosquito kill zone where males are attracted and killed en-masse has been reported [6].The subsequent mass killing of accurately targeted swarms with insecticides resulted in substantial reductions (~80 %) in *An. gambiae* mosquito densities in houses within the Valley de Kou, Burkina Faso [6]. Expectedly, male mosquito research information [12] and specifically male mosquito populations IR data are scanty in literature due to their non-biting habit and exclusion in the standard procedures for IR monitoring [13-16] in mosquito vectors. However, the implementation of male-targeted interventions requires the availability of data on the insecticide susceptibility status of the male mosquito population in the specific locality. It is also crucial to gather evidence regarding the joint susceptibility of male and female mosquitoes to insecticides since both are simultaneously exposed to them in the field. While numerous studies have examined female *Anopheles* [17-20] and *Culex* [21-24] mosquito resistance to insecticides in Nigeria, there is a dearth of research on insecticide resistance in male mosquitoes. This study aimed to address this gap by investigating the insecticide resistance profiles of male and female *An. gambiae* s.l. mosquitoes, both separately and under joint exposure, in Ilorin, Kwara State, Nigeria. The findings will contribute to the possible development of pyrethroid-based attractive toxic sugar baits (ATSB) and swarm control strategies.

2.0 METHODOLOGY

Anopheles mosquito larvae and pupae were collected from breeding sites in Niger (N 080 48.931E'0040 55.416') and Akalambi (N 08050.645'E0040 .55128') areas in Ilorin, Kwara State, North central Nigeria. Collected mosquitoes from the two sites (here after referred to as sites 1 and 2) were transported and reared (27-29 ^{o}C, 77-82% RH) in the insectary at the Department of Zoology, Kwara State University, Malete, Nigeria. Both preadult mosquito types were jointly reared to adulthood using the collection water. Adult male and female *Anopheles* mosquitoes emerging into the same cages were maintained on a 10% sugar solution until exposure.

At least five replicates of 20 to 26 non-blood-fed 3-5-day old adult male and female *Anopheles* were separately exposed to standard WHO Permethrin 0.75%, Deltamethrin 0.05% and Alphacypermethrin 0.05% test papers for 1 hour using WHO test kits and protocol [15].

In the joint exposure susceptibility tests, each replicate of 20-26 mosquitoes exposed to each pyrethroid insecticide

comprised 10-13 each of male and female *Anopheles* exposed together. The 10-13 male and 10-13 female *Anopheles* mosquitoes were transferred together into each exposure tube containing each insecticide test paper. Available adult male and female mosquitoes over each 3-5-day emergence period were allocated to equal numbers of separate and joint exposure replicates. This was done to ensure that replicates of separate and joint male and female mosquito exposures were done at the same time under the same conditions. Numbers of knocked-down mosquitoes were recorded at ten minutes intervals within the 1-hour exposure period. Exposed mosquitoes were maintained on 10% sugar solution in holding tubes for subsequent records of mortality and survival after 24 hours. Two replicates of 20-26 mosquitoes were also exposed to WHO control papers to serve as controls for each category of susceptibility test. Dead and alive mosquitoes were preserved individually over desiccated silica gel in a 1.5ml Eppendorf tube.

Exposed *Anopheles* mosquitoes were morphologically identified using standard identification keys to the species complex level [25]. Subsamples of the *Anopheles* mosquitoes that survived the insecticide exposures were identified to sibling species level. Sibling species of *An. gambiae* s.l were identified using standard PCR [26] and PCR -RFLP [27] procedures. Primers designed from the DNA sequence of the intergenic spacer region of *An. gambiae* s.l; Universal (GTGTGCCCCTTCCTCGATGT), *An. gambiae s.s* (CTGGTTTGGTCGGCACGTTT), *An. arabiensis* (AAGTGTCCTTCTCCATCCTA), *An. merus* and *An. melas* (TGACCAACCCACTCCCTTGA), and *An. quadriannulatus* (CAGACCAAGATGGTTAGTAT) were used For *Anopheles gambiae* s.l. identification. The PCR products obtained were electrophoresed on 1.5% agarose gels, visualized by an ultraviolet transilluminator, and captured in a gel documentation machine.

The time to 50 and 95% knockdown of the exposed mosquitoes (KDT_{50} and KDT_{95}) were determined using a log-time-probit model [28]. For each insecticide, the numbers of male mosquitoes in all the replicates (10-13 per replicate) were added to give the total number of males exposed in the joint exposure. The total number of females tested in the joint exposure per insecticide was also obtained by the addition of female mosquito numbers in all the replicates (10-13 per replicate) conducted for that insecticide. For joint exposures, the percentage mortalities of male and female mosquitoes were determined separately as the number of dead males or females in all the replicates divided by the total number of male or female mosquitoes described above. Percentage mortalities of mosquitoes from male or female-only exposures were determined as numbers of dead mosquitoes in all the replicates of male or female-only exposures divided by the total number of mosquitoes (male or female) exposed per insecticide. The insecticide response status of the mosquito populations was determined as resistant (<90% mortality), suspected resistance (90-97%), or susceptible (≥98%), according to WHO [15]. Pearson's chi-squared test was used to compare the percentage knockdown and mortalities of the male and female mosquitoes exposed to the same insecticides and between separate and joint exposure mortalities.

3.0 RESULTS

Percentage knockdown of male mosquitoes were significantly higher than those of the females exposed jointly to permethrin (Site 1- 63 vs 24%, P<0.001; Site 2- 73 vs 34%, P<0.001), deltamethrin (Site 1- 79 vs 50%, P=0.011; Site 2- 74 vs 46%, P=0.011) and alphacypermethrin (Site 1- 56 vs 31%, P=0.007; Site 2- 78 vs 19%, P<0.001) insecticides (Figure 1).

There were no significant differences between higher percentage knockdown of male and female mosquitoes exposed separately to permethrin (Site 1- 53 vs 51%, P=0.845; Site 2- 63 vs 54%, P=0.405), deltamethrin (Site 1- 41 vs 41%; Site 2- 67 vs 53%, P=0.147) except for alphacypermethrin (Site 1- 62 vs 41%, P=0.039; Site 2- 67 vs 36%, P=0.002) insecticide (Figure 1).

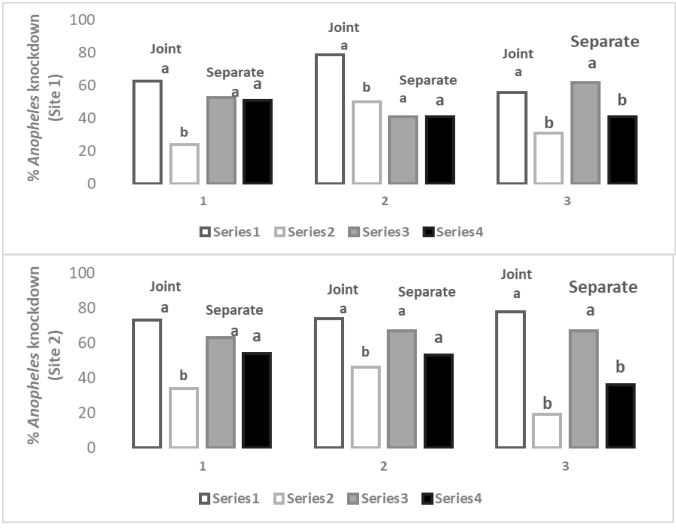


Figure 1. Percentage knockdown of separate and jointly exposed male and female *An. gambiae s.l* mosquitoes from Sites 1 and 2 in urban Ilorin. Knockdown values with different letters under the same insecticide are significantly different (P < 0.05).

Table 1. Knockdown times and percentage mortalities of pyrethroid-exposed male and female *Anopheles gambiae* s.l mosquitoes from Urban Ilorin, Kwara State

Site	Insecticide	Exposure type	Sex of Mosquito	N	KDT_{50} (mins)	KDT_{95} (mins)	Mortality (%)	P value	Status
	Permethrin	Joint	Male	100	42.4	319.6	81	<0.001	Resistant
			Female	113	641.3	4127.5	39		Resistant
		Separate	Male	201	59.6	324.6	75	0.097	Resistant
			Female	170	59.1	323.6	56		Resistant
Site 1	Deltamethrin	Joint	Male	109	22.1	78.2	71	0.680	Resistant
			Female	116	87.3	1888.0	57		Resistant
		Separate	Male	167	57.2	1115.0	60	0.480	Resistant
			Female	230	94.9	2013.0	68		Resistant
	Alphacyper-methrin	Joint	Male	178	45.3	1134.0	86	0.039	Resistant
			Female	109	183.4	16260	61		Resistant
		Separate	Male	136	42.2	418.5	74	0.556	Resistant
			Female	199	91.4	1380.0	67		Resistant
	Permethrin	Joint	Male	186	29.3	203.5	94	0.010	Resistant
			Female	119	212.8	4202	62		Resistant
		Separate	Male	160	46.5	204.3	76	0.067	Resistant
			Female	181	56.6	364.4	55		Resistant
Site 2	Deltamethrin	Joint	Male	155	40.9	230.0	82	0.043	Resistant
			Female	117	60.9	237.8	58		Resistant
		Separate	Male	152	43.4	416.9	69	0.932	Resistant
			Female	167	52.5	435.3	68		Resistant
	Alphacyper-methrin	Joint	Male	209	110.4	5166	75	0.004	Resistant
			Female	146	349.3	11400	44		Resistant
		Separate	Male	448	46.4	623.5	81	0. 077	Resistant
			Female	211	145.1	10960	60		Resistant

N-Numbers of Mosquitoes Exposed, **KDT**-Knock Down Time, mins-Minutes, **P**-Probability Value

The results of *An. gambiae s.l* mosquito knockdown times and percentage mortalities after joint and separate exposures to pyrethroid insecticides are presented in Table 1. Time to 50% knockdown of the male were mostly lower than those of the female mosquitoes exposed to the same permethrin (Male 29.3 - 59.6 vs Female 56.6 – 641.3), deltamethrin (Male 22.1 - 57.2 vs Female 52.5 – 94.9) and alphacypermethrin (Male 42.2 – 110.4 vs Female 91.4 – 349.3) insecticides for both joint and separate exposures of mosquitoes from both sites. Differences in the male and female mosquito mean kdt50 were highest (5-fold) for permethrin (Male 44.5 vs Female 242.5), 1.8-fold for deltamethrin (Male 40.9 vs Female 73.9), and 3-fold for alphacypermethrin (Male 61.1 vs Female 192.3).

Table 2. Sibling Species Composition of Resistant Male and Female *An. gambiae s.l.* Mosquitoes from Different Sites in Urban Ilorin, Nigeria.

Mosquito	Site	Number of samples analysed	*An. gambiae* N (%)	*An. arabiensis* N (%)	*An. coluzzii* N (%)
Male	1	32	4(13.0)	27(84.0)	1(3.0)
	2	31	5(16.1)	23(74.2)	3(9.7)
Female	1	20	3(15.0)	16(80.0)	1(5.0)
	2	29	4(13.8)	23(79.3)	2(0.0)

N-Numbers of mosquitoes identified for each species.

All male mosquito populations were resistant to permethrin (75-94% mortality), deltamethrin (60-82%), and alphacypermethrin (74-86%) insecticides. Higher levels of resistance were found in the female mosquitoes, which elicited lower mortality responses to the permethrin (39-62%), deltamethrin (57-68%), and alphacypermethrin (44-60%) insecticides.

The percentage mortalities of male mosquitoes mainly were significantly ($P \leq 0.043$) higher than those of the jointly exposed females (Table 1). For separate exposures, higher male mosquito mortalities observed were not significantly ($P \geq 0.067$) different from those of the females.

Male mosquito mortalities from joint and separate exposures to permethrin (Site 1- 81 vs 75%, P=0.631; Site 2- 94 vs 76%, P=0.167), deltamethrin (Site 1- 71 vs 60%, P<0.337; Site 2- 82 vs 69%, P=0.290) and alphacypermethrin (Site 1- 84 vs 76%, P=0.343; Site 2- 75 vs 81%, P=0.631) insecticides were not significantly different (Figure 2). There were also no significant differences in the female mortalities from joint and separate permethrin

(Site 1- 39 vs 56%, P=0.081; Site 2- 62 vs 55%, P=0.518), deltamethrin (Site 1- 57 vs 68%, P=0.325; Site 2- 58 vs 68%, P=0.373) and alphacypermethrin (Site 1- 61 vs 67%, P=0.596; Site 2- 44 vs 60%, P=0.117) exposures (Figure 2). The resistant male and female *An. gambiae s.l* mosquitoes were predominantly of the *An. arabiensis* (74-84%) species compared to lower proportions of *An. gambiae* (13-16%) and *An. coluzzii* (0-9%) species (Table 2).

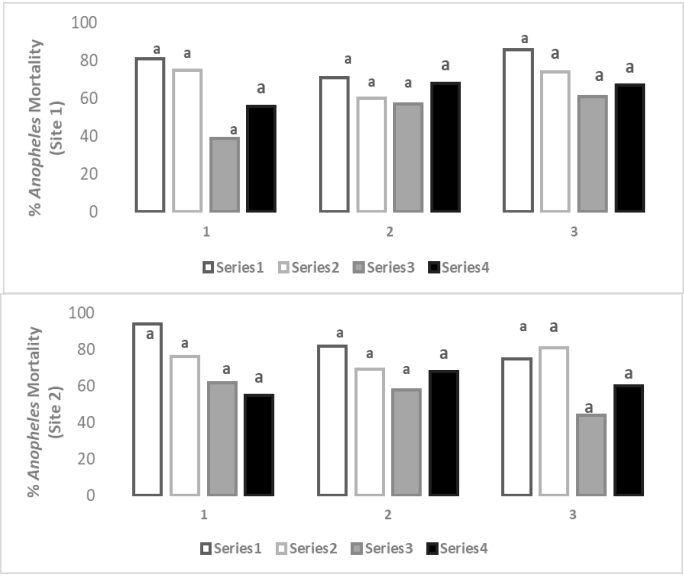


Figure 2. Percentage mortality comparisons of males in separate and joint exposures and females *An. gambiae s.l* in separate and joint exposures. Percentage mortality values with same letters under the same insecticide are not significantly different (P > 0.05).

4.0 DISCUSSION

This study provides evidence of multiple pyrethroid insecticide resistance in male and female *An. gambiae s.l* mosquitoes, assessed both in separate and joint exposures. The knockdown and mortality rates were higher in male mosquitoes compared to females. However, the unexpected finding in this study was the lack of significant differences in the percentage knockdown between male and female mosquitoes and the percentage mortality in separately exposed male and female mosquitoes. To date, WHO insecticide susceptibility test procedures exclude males from resistance monitoring experiments [16], partly due to the expectation for heightened insecticide susceptibility compared to females [15,16]. Significantly higher mosquito mortality is expected among males than females due to their smaller size, increased fragility, and shorter life expectancy [15]. However, the findings of this study reveal confirmed male mosquito resistance to all tested pyrethroid insecticides, both in separate and combined exposures, contradicting the notion of heightened insecticide susceptibility in male mosquitoes. The intrinsic attributes that enhance male mortality may have been countered by the phenotypic insecticide resistance observed in the male mosquitoes in this study. The confirmed resistance status of the males in separate and joint exposures suggests that the mosquitoes may have been similarly impacted by environmental insecticide selection pressure as the female. Stewart et al. [9] demonstrated the successful use of ITN and indoor ATSB which could be deployed in both rural and urban communities. However, multiple insecticide resistance found in the male and female mosquitoes imply the potential ineffectiveness of pyrethroid-based ATSB, ITNs, and swarm spraying to achieve reductions in the male population, female mating, and overall mosquito vector population density in the present urban study location. *Culex* is less amenable, especially to pyrethroid insecticides [29,30]. Indeed, suspected *Culex* male mosquito deltamethrin resistance has been reported earlier in south-eastern Tanzania [7]. However, the present study, to our knowledge, represents the first report on multiple phenotypic pyrethroid insecticide resistance evidence in the male *An. gambiae s.l* mosquitoes. Male *Anopheles* resistance found in this study is attributable to urban control and domestic use of insecticides and other products with similar active ingredients probably impacting on the larval breeding sites with attendant insecticide resistance selection pressure on both male and female mosquito larvae. Evidence of LLIN utilization resulting in malaria vector resistance over time has also been documented in different malaria-endemic settings [31,32]. Pyrethroid-based vector control involving massive ITN distribution and utilization in this urban locality over the years [33,34] could also have contributed to insecticide resistance selection pressure on the mosquitoes, triggering the development of resistance detected in this study. Apart from that of Awolola *et al.,* [17], our study represents another report of multiple pyrethroid resistance found in Nigeria's female urban *An. gambiae s.l* populations. Other recent pyrethroid resistance monitoring reports involving urban female *Anopheles* populations were limited to only permethrin [35,36], deltamethrin [19,22,37], or both [18,38], leaving out alphacypermethrin. This study found significantly higher male than female mosquito knockdown from separate exposures of both mosquitoes to permethrin and deltamethrin but not alphacypermethrin.

This resulted from markedly lower alphacypermethrin knockdown of females compared to the male mosquitoes and suggests significantly lower alphacypermethrin sensitivity in the females than male mosquitoes. Alphacypermethrin testing is crucial, especially now that it is one of the active ingredients incorporated into some of the newly introduced dual active ingredients (AI) ITNs. A first report on joint male and female *Anopheles* mosquito insecticide susceptibility testing is also presented in this study. In contrast with separate exposures, significantly higher male knockdown and mortalities than female was recorded from the joint male and female mosquito exposures. This result could have emanated from the male smaller size and fragility effects becoming more pronounced during their exposures with the stronger and larger females than in the separate exposures where only male mosquitoes were exposed alone. Other studies conducted in rural communities in Kwara state have also reported the prevalence of *An. gambiae* over *An. coluzzii* but with the negligible occurrence of *An. arabiensis* [39-41] except in cattle-rearing communities [42-43]. The preponderance of *An. arabiensis* in Ilorin city, the Kwara state capital, aligns with the urban availability of several temporary sunlit mosquito breeding pools preferred by the species. This study has established separate and joint male and female pyrethroid insecticide resistance status of *An. gambiae s.l* mosquitoes in the study location. Further studies should investigate resistance mechanisms in both mosquito sexes for proper resistance management to restore the susceptibility of the mosquitoes to the widely used pyrethroid insecticides.

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Conflicts of Interest

The authors declare that there is no conflict of interests.

Authors' Contributions

AO conceived and designed the study, conducted bioassays and wrote the manuscript. **FY, RB, RA, IM** conducted bioassays. **TO, AOA** contributed data analysis tools. **YB , NI, OA** participated in larval collection. All authors approved the final copy of the manuscript.

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