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Impact of Urban Agriculture on the Species Distribution and Insecticide Resistance Profile of *Anopheles gambiae s.s.* and *Anopheles coluzzii* in Accra Metropolis, Ghana

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Abstract

Malaria incidence in urban areas has generally been low compared to rural areas but recent data indicate that urban malaria remains a public health problem. It is therefore important to understand the factors that promote urban malaria to help formulate future vector control strategies. This study compared *Anopheles gambiae s.l.* (*A. gambiae s.l.*) species composition, distribution and insecticide resistance mechanisms between vegetable and non-vegetable growing areas in Accra Metropolis. Four sites were selected within the city of Accra which comprised of two vegetable-growing and two non-vegetable growing areas. WHO susceptibility tests were carried out on adults *A. gambiae s.l.* reared from larvae collected from the sites. Five insecticides were tested and the *A. gambiae* complex, resistance genotypes and enzyme activities of each population were characterized. All *A. gambiae s.l.* populations tested were resistant to all the insecticides, but relatively lower mortalities were observed in the vegetable growing areas. The mortality against 0.05% deltamethrin was 2.6% (Opeibea) and 12.5% (Korle-Bu) for the vegeta-

ble growing areas and 36.2% (Achimota) and 38.9% (Mataheko) in the non-vegetable growing areas. *Anopheles gambiae s.s.* (95% of Opeibea population) and *Anopheles coluzzii*, (98% of Korle-Bu population) were the dominant species in the vegetable growing areas. The voltage-gated sodium channel (*Vgsc*-1014*F*) frequencies of all the populations were similar but the acetylcholinesterase (*ace*-1) frequencies were significantly lower ($p < 0.05$) in Korle-Bu and Mataheko populations. High level of P450s and esterases were observed in the *A. gambiae s.l.* from Opeibea than from the other areas. The contribution of urban agriculture in the development of insecticide resistance needs to be considered in the formulation of future vector control strategies alongside other domestic usages.

Keywords

Anopheles gambiae s.s., *Anopheles coluzzii*, Insecticide Resistance, Malaria, Piperonyl Butoxide (PBO), Urban Agriculture

1. Introduction

Urban agriculture is a widely practiced farming activity involving more than 800 million people, worldwide [1]. Growing population and rural migration to the cities of African countries have resulted in the practice of urban and peri-urban agriculture to provide food security, to overcome poverty and create employment. Although most of these urban inhabitants are engaged in subsistence gardening, more than 200 million practice market-oriented farming on undeveloped urban spaces [2].

The same trend is observed in Ghana where the population of Accra metropolis has grown from 1.6 million people in 2000 to about 2.27 million in 2015 [3]. While it is critical meeting urban challenges such as food security and unemployment, these practices also present a serious public health problem [4]. The economic and social value of urban and peri-urban agriculture is hindered by a number of factors including the proliferation of mosquito breeding sites [5] [6] with potentially higher malaria epidemiological risk [7]. Malaria transmission in urban settings such as in Accra is influenced by several factors including housing, availability of health care, knowledge on vector control strategies and vector susceptibility to insecticides.

Intensive use of insecticides for both public health and agricultural pest control has led to reports of global spread of insecticide resistance. The trend is threatening the efficacy of malaria vector control tools and even worsened by the use of the same insecticide or class of insecticides [4] [8] [9] [10] [11]. Ghana is not exempted from this phenomenon particularly where pyrethroid resistance has been reported in many areas in the country [12] [13] [14] [15]. Recently, there is a scaling up of Long Lasting Insecticidal Net (LLIN) distribution and Indoor Residual Spraying (IRS) coverage in the country [16]. Resistance to py-

rethroids by malaria vectors is known to be driven by selection pressure resulting from the increased use of LLINs and IRS [11] [17] and has also been attributed to the contamination of mosquito breeding habitats during the application of agricultural pesticides. Agricultural environments can create mosquito breeding habitats, especially when irrigated, thereby increasing vector density [4] [8] [10]. *Anopheles gambiae s.s.* and *Anopheles coluzzii* constitute the main malaria vectors in Ghana and particularly in the Southern part of the country where Accra is located. *Anopheles gambiae s.s.*, (formerly named *A. gambiae s.s.* molecular form) prefers rain-dependent pools, sunny and temporally puddles while *A. coluzzii* (formerly *A. gambiae s.s.* M molecular form) exploits preferentially immature sites that exist across seasons and also linked to human activities such as rice cultivation, urbanization and irrigation [13].

Malaria incidence in urban areas has generally been low compared to rural areas. However, data collected throughout the decade indicate that urban malaria remains a major public health problem [7] [18] [19] [20] [21] [22]. It is therefore important to understand the factors that promote urban malaria to help formulate vector control strategies and help manage insecticide resistance in urban areas. The aim of this study was to determine the distribution of malaria vectors and the contribution of urban agriculture to the development of insecticide resistance in some selected areas within Accra Metropolis.

2. Material and Methods

2.1. Study Sites

The study was conducted in Accra, the capital of Ghana (5˚32'59.99"N and 0˚12'60.00"E). Accra is the country's biggest metropolis, most diverse and most cosmopolitan with an estimated population of 2.27 million of people as in 2015. Accra is in the coastal savanna ecological zone, characterized by two rainfall peaks, the first occurring from April to July and the second from September to October. The region receives an average rainfall of 740 mm to 890 mm per year. The lowest mean monthly temperature (about 26˚C) is recorded during August and the highest (about 30˚C) between March and April. The mean relative humidity throughout the year ranges between 65% and 75%.

For the study, most of the urban and peri-urban vegetable growing areas were mapped using a 3D global positioning system (GPS) to determine the specific geographical coordinates or geo-location of the sites. Two vegetable growing sites were selected among them and paired with two non-vegetable growing areas close to each vegetable growing site and served as controls. The sites included Korle Bu (5˚32'20.124"N; 0˚14'10.115"E) and Opeibea (5˚35'54.54"N; 0˚10'53.609"E) as vegetable growing sites and Mataheko (5˚34'0.4"N; 0˚15'13.8"W), and Achimota (5˚36'59.99"N; 0˚13'60.00"E) as non-vegetable growing sites (**Figure 1**). A questionnaire survey on insecticides and herbicides classes and type was conducted to determine the extent of usage of insecticides.

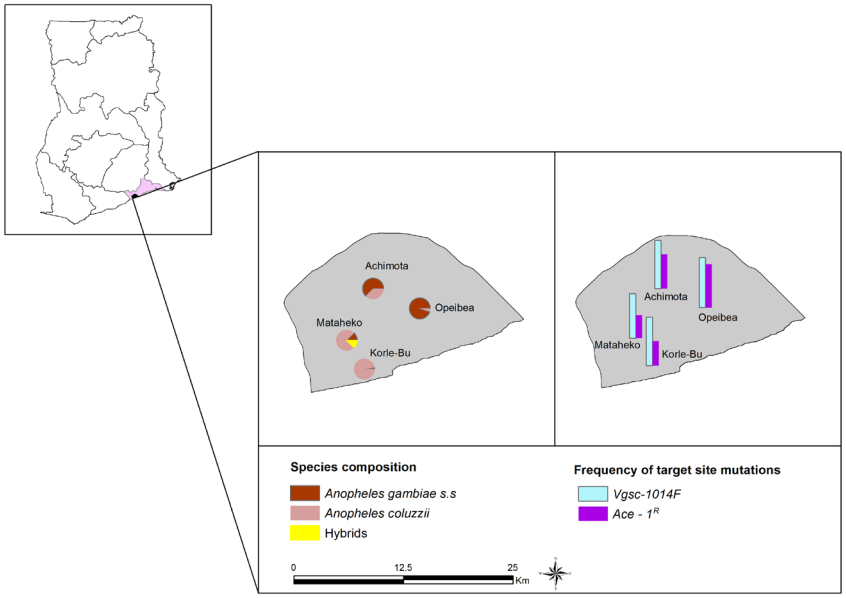

Figure 1. Map of the study sites including the *A. gambiae s.l.* species composition and the frequencies of the target site mutations within each population.

2.2. Mosquito Sampling and WHO Susceptibility Test

Larvae and pupae were collected from the study sites between January and April 2017. The mosquito larvae were reared to adults in the insectary of Vestergaard-NMIMR Vector Labs (VNVL) at the Noguchi Memorial Institute for Medical Research (NMIMR) using standard rearing methods [23].

Susceptibility tests of WHO were conducted according to WHO standard protocols (WHO, 2016). Mosquitoes were assayed using WHO discriminating dosages of five insecticides: 0.05% deltamethrin, 0.75% permethrin, 4% DDT, 5% malathion and 0.1% bendiocarb. The insecticides were selected to match with the four classes of insecticides. In addition, synergist assays with 4% Piperonyl butoxide (PBO) impregnated papers were conducted to determine the probable presence of metabolic mechanisms such as P450 enzymes. Twenty to twenty-five non-blood fed female *A. gambiae s.l.*, aged 3 - 5 days were exposed for one hour to the different insecticides before and holding period of 24 hours for mortality. For the synergist assay, mosquitoes were pre-exposed to PBO for one hour before exposure to the insecticide for an additional hour. The number of mosquitoes knocked down was recorded at 60 minutes and mortality recorded after 24 hours post exposure for both tests. Tests with silicone and olive oil impregnated papers were run in parallel and served as controls. After the susceptibility tests, all mosquitoes (including controls) were kept at −20˚C for further identification of *A. gambiae* species complex and characterization of the voltage-gated sodium channel 1014*F* (*Vgsc*-1014*F*) and acetylcholinesterase *G*119*S* (*ace*-1) mutations.

2.3. DNA Extraction and Species Characterization

Forty mosquito samples kept at −20 ˚C after WHO susceptibility test were ran-

domly selected per site for molecular characterization. The whole DNA of every single mosquito was extracted using a modified CTAB method described by Collins *et al*. [24]. Thereafter, the *A. gambiae* species identification was done using the protocol of Fanello *et al*. [25] and the primers UN [GTGTGCCGCTTCCTCGATGT], AG [CTGGTTTGGTCGGCACGTTT], AR [AAGTGTCCTTCTCCATCCTA] and AM [GTGACCAACCCACTCCCTTGA] and Santolamazza *et al*. [26] with primers F6.1a [5'-TCGCCTTAGACCTTGCGTTA-3'] and R6.1b [5'-CGCTTCAAGAATTCGAGATAC-3']. The *Vgsc*-1014*F* and *ace*-1 allele of each mosquito were characterized following the protocol of Martinez-Torres *et al*. [27] using the primers AGD1 [5'-ATAGATTCCCCGACCATG-3']; AGD2 [5'-AGACAAGGATGATGAACC-3'], AGD3 [5'-AATTTGCATTACTTACGACA-3'] and AGD4 [5'-CTGTAGTGATAGGAAATTTA-3'], and Weill *et al*. [28] with primers EX3AGdir [5'-GATCGTGGACACCGTGTTCG-3'] and EX3AGrev [5'-AGGATGGCCCGCTGGAACAG-3'] respectively.

Furthermore, 50 non-exposed mosquitoes per site were kept at −85˚C and the enzyme activities of each population were characterized following the methods described in MR4 [23], and compared with the susceptible strain of *A. gambiae* Kisumu. These enzymes included oxidases (P450s), esterases (α and β), acetylcholine esterase (Ache), proteins and Glutathione S-Transferases (GSTs).

2.4. Data Analysis

The resistance status of each *A. gambiae* mosquito population was determined following the WHO criteria, where mortality ≤ 90% indicates that the colony is resistant to the insecticide tested, >90% and <97%, the resistance is suspected and ≥98%, the colony is susceptible (WHO, 2016).

The *Vgsc*-1014*F* and *ace*-1 frequencies were calculated using Hardy Weinberg formula and compared among populations using the z-test of proportion.

The enzyme activities were estimated as the amount of product per minute per milligram of protein. The mean values were plotted and compared using the non-parametric test of Kruskal Wallis of the Graph Pad Prism 5.0 software.

3. Results

3.1. Insecticide Usage and *A. gambiae s.l.* Resistance Status

The results of the insecticide usage survey are described in **Appendix**. Almost all the classes of insecticides were found within the pesticides and herbicides been used for controlling pests in the vegetable growing areas. However, the organophosphates were the most common insecticides found followed by the pyrethroids.

The WHO susceptibility test results are described in **Figure 2**. The resistance to deltamethrin was significantly higher in Opeibea and Korle-Bu (2.6% and 5.7% respectively), the vegetable growing sites than the non-vegetable growing

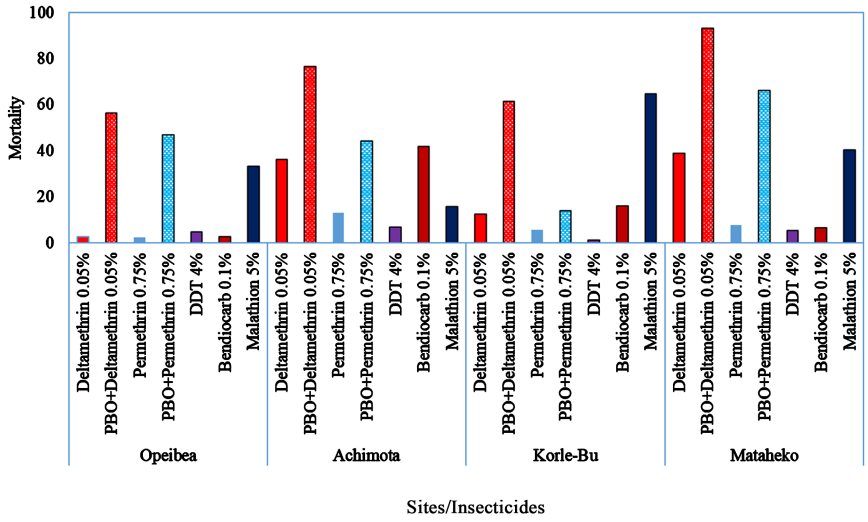

Figure 2. Mortality of *A. gambiae s.l.* of the different sites against various insecticides.

sites Achimota and Mataheko (36.2% and 38.9% respectively) ($p < 0.05$). A similar trend was observed with permethrin though the mortalities were relatively lower than deltamethrin in all the sites. The mortalities against permethrin ranged between 2.5% and 13.1% for both vegetable and non-vegetable sites. When *A. gambiae s.l.* was pre-exposed to PBO, a significant enhancement of susceptibility was observed with both insecticides and in all the sites. However, the synergistic action of PBO was significantly higher for deltamethrin than permethrin in all the sites. The mortalities against PBO + deltamethrin in the vegetable growing sites were 56.5% and 61.5% for Opeibea and Korle-Bu respectively, while PBO + permethrin showed mortalities of 46.8% and 14% respectively in the same sites. Similarly, the non-vegetable sites Achimota and Mataheko showed 76.5% and 93.2% for PBO + deltamethrin respectively compared to 44.1% and 66.2% for PBO + permethrin respectively. All the populations of *A. gambiae s.l.* from all the sites were highly resistant to DDT with mortalities ranging between 1.2% at Korle-Bu and 7% in Achimota. The other insecticide classes including the carbamate (bendiocarb) and organophosphate (malathion) recorded high resistance in all the sites. Though the mortality against bendiocarb was low, malathion in contrast recorded a relatively higher mortality of 64.6% at Korle-Bu.

Overall, no significant difference was observed between the percentage mortalities of the mosquitoes exposed to insecticides across sites ($p = 0.493$).

3.2. Molecular Characterization of *A. gambiae s.l.* Populations of the Sites

The results of the species identification and resistance allele mutations are shown in Figure 1. The majority (95%) of *A. gambiae s.s.* occurred in Opeibea compared to 97.5% of *A. coluzzii* in Korle-Bu. The *Vgsc*-1014*F* frequency of *A. gambiae* in Opeibea and Korle-Bu were similar (0.91 vs 0.88). In contrast, the *ace*-1 mutation was significantly higher ($p = 0.001$) within the population of

Opeibea (0.79) than Korle-Bu (0.45).

For the non-vegetable growing areas, the populations were living in sympatry with *A. gambiae s.s.* and *A. coluzzii* constituting 62.5% and 37.5% respectively in Achimota. In Mataheko, *A. coluzzii* and *A. gambiae s.s.* formed 75% and 12.5% of the species respectively. Hybrids of both species (12.5%) were found in Mataheko. The frequency of the *Vgsc*-1014*F* is also similar in both non-vegetable growing sites with 0.88 and 0.81 observed in Achimota and Mataheko respectively. The *ace*-1 frequencies were 0.63 for Achimota and 0.42 for Mataheko.

3.3. Biochemical Analysis

The biochemical assays showed the involvement of some enzymes in the detoxification of insecticides as described in Figure 3 and Table 1. The population of

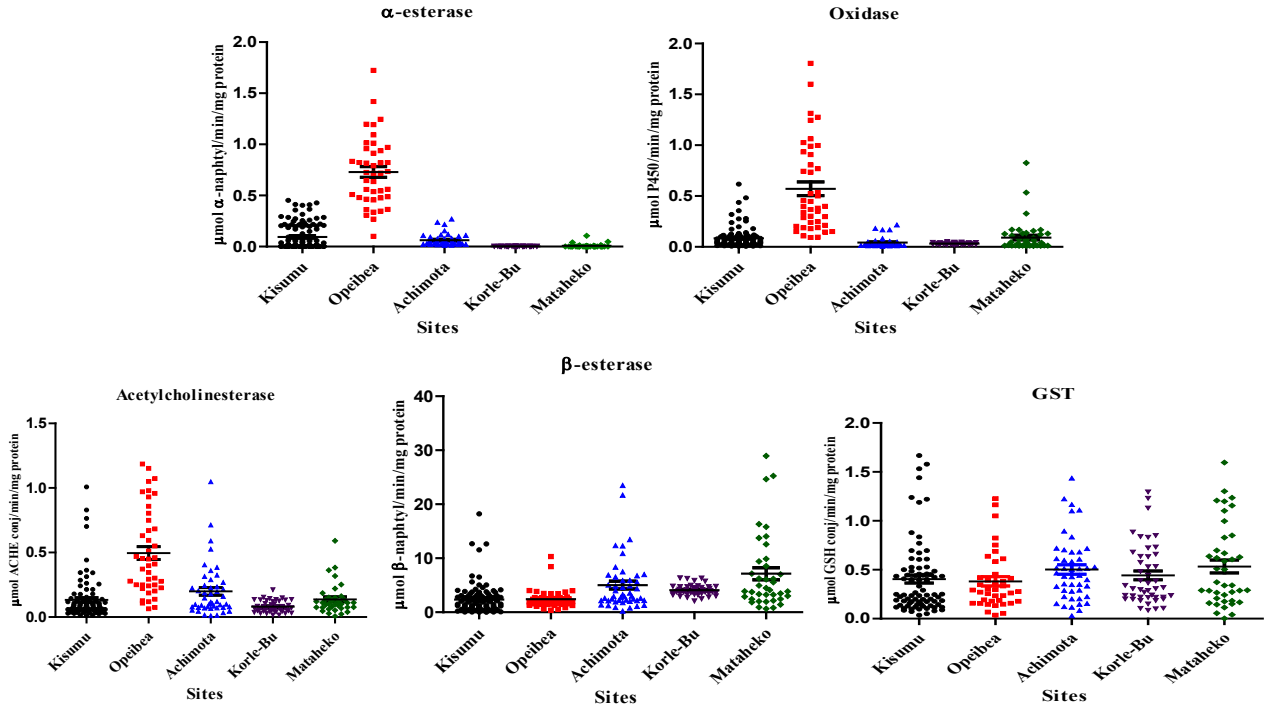

Figure 3. Comparison of the different enzyme activities of *A. gambiae s.l.* from the study sites.

Table 1. Enzyme activities of *A. gambiae s.l.* populations of the different sites compared to the susceptible *A. gambiae s.s.* Kisumu.

Mosquito colony	Oxidase (Mean P450 activity) in µmol	*p value*	*α*-esterase (Mean *α*-naphtyl) in µmol	*p value*	*β*-esterase (Mean *β*-naphtol activity) in µmol	*p value*	Ache (Mean Ache conj. activity) in µmol	*p value*	GST (Mean GSH conj. activity) in µmol	*p value*
Kisumu	0.08495 ± 0.01956		0.09495 ± 0.02435		2.358 ± 0.551		0.1330 ± 0.1901		0.4042 ± 0.0811	
Opeibea	0.5716 ± 0.1371	0.001	0.7299 ± 0.1067	0.005	2.424 ± 0.549	0.073	0.4958 ± 0.2099	<0.0001	0.3814 ± 0.0955	0.39
Achimota	0.04169 ± 0.01394	**<0.0001**	0.06291 ± 0.01716	**0.028**	5.024 ± 1.439	<0.0001	0.1989 ± 0.2074	0.019	0.5028 ± 0.0965	0.045
Korle-Bu	0.03312 ± 0.00205	**<0.0001**	0.002638 ± 0.01716	**<0.0001**	4.086 ± 0.271	<0.0001	0.08021 ± 0.2705	0.378	0.4420 ± 0.0912	0.252
Mataheko	0.09098 ± 0.04533	**0.001**	0.007071 ± 0.005323	**<0.0001**	7.137 ± 2.269	<0.0001	0.1374 ± 0.2629	0.091	0.5331 ± 0.1328	0.015

Degree of significance of the *p* value is 5%. (*p* value in bold are significantly lower than the reference *A. gambiae* Kisumu).

A. gambiae s.l. from Opeibea recorded the highest level of enzyme activity compared to the other sites. P450s, α-esterase and acetylcholinesterase were significantly higher in Opeibea ($p < 0.05$) than both non-vegetable growing sites and Korle-Bu populations. However, the expression of β-esterase activity was relatively higher mostly in the *A. gambiae s.l.* population of the non-vegetable growing areas.

4. Discussion

Farming activities in West African urban areas (capitals and big cities) have an important impact on the density of mosquito populations [5] [29] [30]. Nowadays, urban and peri-urban vegetable growing activities contribute significantly to the gross domestic product (GDP) of countries and serve as sources of income for citizens who are engaged in these activities. Nonetheless, these urban vegetable-growing activities involving indiscriminate use of insecticides has led to the creation of favorable breeding sites for *Anopheles* mosquitoes and the development of insecticide resistance. These are major factors that contribute to urban malaria transmission in cities such in Accra [31].

In this study, high insecticide resistance of *A. gambiae s.l.* to all the classes of insecticides was observed in the vegetable growing areas and particularly to pyrethroids and organochlorine (**Figure 2**). The resistance to deltamethrin and permethrin was more than ten-fold greater in the vegetable growing areas (Opeibea and Korle-Bu) than thenon-vegetable growing areas (Achimota and Mataheko).

This observation confirms work done by Yadouleton *et al*. in Benin and Kabula *et al*. in Ghana [4] [32] showing high insecticide resistance of *A. gambiae s.l.* within urban vegetable growing areas. Additionally, the demand of freshly produced vegetables by the urban market has increased the development of vegetable growing spaces and the use of insecticides and pesticides. These factors might have contributed to the development of insecticide resistance of *A. gambiae s.l.* mosquitoes that is observed. However, the synergistic effect using Piperonyl butoxide (PBO) and deltamethrin was very effective in enhancing susceptibility of these resistant mosquitoes, thereby increasing the mortality of *A. gambiae s.l.* collected from both vegetable and non-vegetable growing areas. Nevertheless, there were differences in the percentage increment of susceptibility by PBO in both collection sites. In the vegetable growing areas, the mortalities were increased by five and twenty folds using PBO + deltamethrin in Korle-Bu and Opeibea respectively while it doubled in the non-vegetable growing areas (Mataheko and Achimota).

The same trend was observed with PBO + permethrin where the percentage of susceptibility enhancement in *A. gambiae s.l.* from Opeibea, a vegetable growing area was significantly higher compared to the non-vegetable growing sites. The results also showed low level of enhancement of susceptibility when mosquitoes from Korle-Bu were pre-exposed to PBO before permethrin compared to Opeibea. PBO + permethrin showed three folds enhancement of susceptibility in *A.*

gambiae s.l. from Korle-Bu, whilst that of Opeibea was about twenty folds (**Figure 2**). The difference observed in the ability of PBO to increase the mortality of the two populations of *A. gambiae s.l.* might be due to difference in mosquito species composition and the non-involvement of metabolic resistance mechanisms. *Anopheles coluzzii* was abundant at Korle-Bu while *A. gambiae s.s.* dominated the population of Opeibea (**Figure 1**). Furthermore, the latter species was observed to exhibit higher P450 enzyme activity than *A. coluzzii* from this study (**Figure 3**, **Table 1**) and this observation also confirms the fact that *A. gambiae s.s.* was more resistant than *A. coluzzii* [33] [34]. Piperonyl butoxide (PBO) is known as a synergist which acts as an inhibitor of the P450 enzyme activities [35].

The level and probable involvement of P450s in the resistance status of the mosquitoes from all the study sites have been first described using the synergistic effect of PBO [36] and was further confirmed by the characterization of the level of the different enzyme activities including P450s, esterases, Glutathione S-transferase (GST) and acetylcholine esterase (Ache). The high level of P450s activities observed mainly in *A. gambiae s.l.* from Opeibea confirmed the involvement of oxidases in the insecticide resistance level of the mosquitoes (**Figure 3** and **Table 1**).

Relatively high resistance to other insecticides including bendiocarb and malathion was observed in both vegetable and non-vegetable growing areas. The slightly high level of resistance observed in the non-vegetable growing areas is an indicator of the spread of insecticide resistance which may be sustained by the household use of vector control products such as mosquito coils and aerosols as well as insecticide treated nets. The study also showed that the two major malaria vectors *A. gambiae s.s.* and *A. coluzzii* occurred in sympatry though in significantly different proportions within the various sites (**Figure 1**). The two species were specially found in larger proportion in the vegetable growing areas with *A. gambiae s.s.* representing more than 95% of the total population in Opeibea while 98% of the *anopheles* from Korle-Bu were *A. coluzzii*. The difference in the species composition could be due to the geographical position of the two vegetable growing sites. Korle-Bu is located at a lower altitude than Opeibea and the vegetation is relatively dense while Opeibea is a sunny and opened area. The difference in the ecological niche might have shaped their adaptation since *A. gambiaes.s.*is more adapted to sunny areas than *A. coluzzii* [33]. In addition, the species distribution in the non-vegetable growing areas seemed to have been driven by the proximity of the population to the vegetable growing sites. A higher proportion of *A. gambiae s.s.* was observed at Achimota which was close to Opeibea, while Mataheko showed high percentage of *A. coluzzii* due to its proximity to Korle-Bu. However, this observation need to be further investigated and confirmed using a genomic tree to characterize the different population structures and their association [37].

The voltage-gated sodium channel (*Vgsc*-1014*F*) which is known to confer resistance to pyrethroids and DDT was highly observed in all the sites including

the non-vegetable growing areas (**Figure 1**). This is a call for concern since this observation is indicative of widespread of *Vgsc* mutation within the *A. gambiae s.l.* populations in Accra. Over the years, various studies have shown gradual increase of the frequencies of *Vgsc* mutation in *Anopheles gambiae s.l.* from Accra and the whole country [12] [13] [15] [32] [38] and probably sustained by the intense use of pyrethroids in most of the vector control tools. It is therefore not surprising that high frequencies of resistance alleles were observed in the non-vegetable growing sites purported to be residential areas.

The *ace*-1 mutation, known to confer resistance to carbamates and organophosphates showed a relatively higher frequency in vegetable growing areas than the non-vegetable growing sites ($p < 0.05$). This could be due to the massive use of carbamate and organophosphate-based pesticides to control agricultural pests as confirmed by the results of the pesticide use survey in the study areas (**Appendix**). It is therefore important to properly manage urban vegetable growing activities and use of pesticides to prevent further spread of insecticide resistance and eventual failure of vector control strategies using these insecticides. The use of insecticides to control pest in agriculture obviously contributes to the spread of insecticide resistance of malaria vectors [39], which was also observed within urban and peri-urban vegetable growing activities in Accra. Therefore, the monitoring of insecticide resistance and associated mutations in vector populations are relevant for early detection and prompt decision making by appropriate authorities.

5. Conclusions

The distribution of *A. gambiae s.l.* indicated that both *A. gambiae s.s.* and *A. coluzzii* occurred in sympatry in both study areas. The *Vgsc*-1014*F* is highly and widely spread within the *A. gambiae s.l.* populations in Accra, irrespective of whether the site is a vegetable growing area or not. The *ace*-1 was also found in both settings but in a significantly higher proportion within the vegetable growing areas. Agricultural activities using pesticides and household vector control influence insecticide resistance in malaria vector populations. The findings of this study will be useful in understanding the contribution of urban agriculture to the increase in urban malaria transmission and help formulate policies for future vector control in urban areas.

Limitation: While the study was able to emphasize the impact of the use of agricultural pesticides on the resistant status of the malaria vectors within Accra, it was in contrast difficult to estimate the correlation between that trend and household use of protective measures using long lasting insecticidal nets (LLINs) and other treated materials.

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Authors' Contributions

JC, KKF and SKD designed, implemented the study, analyzed, interpreted data and drafted the manuscript. MCE, RP, JJ, DO, GA, DAB, CA, AI, SA, and SG carried out mosquito collections and all laboratory experiments. SDK, KKF, MPH and HPJ revised the manuscript. All authors read and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

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Appendix

Table A1. Class and active ingredient of agrochemicals used by the farmers in both vegetable growing areas.

Agrochemicals	Class	Active ingredients
Protect 1.9 EC	Organophosphate	Emamectin Benzoate 1.92% EC
Confident 532 EC	Pyrethroid + Neonicotinoid	Cypermethrin + Endosulphan
Bypel 1 (PrGV-Bt)	Biopesticide	Pierixrapaedarnulosis virus 10,000 pib/mg + Bacillus thurinbiensis 15,000 µ/mg
Attack (EC)	Pyrethroid	Pirimiphos-methyl 475 g/L + Permethrin 25 g/L
Lambda super 2.5 EC	Pyrethroid	Lambda Cyhalothrin 5% EC
Akape (Anti-Attah)	Organophosphate	Imidacloprid
Porselen	Avermectin	Emamectin Benzoate 5% SG
Agricombi	Organophosphate	Fenitrothion 30% + Fenvalerate 10%
Agrithane 80 WP	Fungicide	Mancozeb 800 g/kg
Cydimsuper	Pyrethroid + Organophosphate	Cypermethrin + Dimethoate
Dursban	Organophosphate	Chlopyrifos
Goland (SL)	Neonicotinoid	Acetamiprid