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Larval Productivity and Detoxification Enzymes Profile in Response to Physico-chemical Environmental Factors of *Anopheles gambiae* Breeding Ecologies in Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author AAI designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Author YD managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: The aim of this study is to investigate *Anopheles gambiae* larval tolerance, density- as a function of survivorship- and the response of their detoxification enzymes to levels of various physico-chemical environmental factors present in their breeding sites.
Study Design: Mosquito breeding sites were grouped into three different study zones A, B & C on the basis of human related activities (intensive agriculture, petrochemical industries and domestic activities, respectively) taking place within and/or around the breeding sites, followed by sampling of *An. gambiae* larvae and determination of larval density from all the breeding sites across the

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designated study zones. Some of the sampled larvae were reared into pupae and adult. Levels of 7 physical (pH, temperature, conductivity, transparency, total dissolved solids, dissolved oxygen and biological oxygen demand) and 6 chemical (sulphates, phosphates, nitrites, nitrates, carbon content and oil and grease) environmental factors were determined from mosquito breeding sites across the three study zones. Activities of the 3 major detoxification enzymes (P450, GST and α & β-esterases) were assayed on the sampled larvae and the emerged pupae and adult.

Results: Our results showed high degree of tolerance of *An. gambiae* larvae to higher levels of these environmental factors. Also, the activities of the detoxification enzymes were higher in study zones A & C (which also recorded higher levels of the environmental factors) and were highly associated with most of the physico-chemical environmental factors. A deduced statistical model established the chemical composition in combination with some of the physical environmental parameters as influencing factors for larval density and producing an inductive effect on the three detoxification enzymes across the three life stages.

Conclusion: These observations could have a significant impact on the environmental management and insecticide-based approach to vector control in Nigeria.

Keywords: Anopheles gambiae; environmental factors; breeding ecologies; detoxification enzymes.

1. INTRODUCTION

According to the world health organization (WHO), about half of the world population (3.3 billion) is at risk of malaria. Although the past several years have witnessed tremendous increase in control measures, malaria still remains the number one killer disease especially in sub-Saharan Africa. More than 216 million cases were reported in 2010 alone, with over 660, 000 deaths recorded [1]. In Nigeria, the entire population of over 170 million is at risk of malaria which is responsible for about 60% and 30% of outpatients' visits and hospitalizations respectively [2]. Insecticide-based control measures such as Long Lasting Insecticide-treated mosquito Nets (LLINs) and Indoor Residual Spraying (IRS) are widely used to control malaria vector. These measures are currently inadequate [3] and not sufficient to halt malaria transmission and would likely contribute to the eventual emergence of insecticide resistant mosquitoes [4,5,6]. Currently researchers have been exploring several alternative avenues of controlling malaria, and one particular approach that appears to be gaining attention is an environmental management strategy that aims to reduce adult vector population by targeting their aquatic immature stages (i.e., mosquito eggs, larvae and pupae). This strategy is becoming increasingly important in many countries especially in sub-Saharan Africa and involves different species of mosquitoes including those that transmit malaria. The strategy depends on the use of various larvicidal techniques and environmental management practices aimed at reducing larval density and therefore minimizing or reducing vector abundance [7].

However, since mosquito breeding sites are found in various environments ranging from farmlands to sites of industrial activities, deliberate human intervention at controlling mosquito populations may not be the only contribution to the factors affecting larval growth and development. For instance, some of the most active breeding sites for mosquitoes are located around farmlands where various agro-allied chemicals such as fertilizers, pesticides and herbicides, are applied to enrich soil and control agricultural pests and diseases [8,9,10]. Active mosquito breeding sites have also been reported in areas polluted by industrial effluents, rotting vegetation, human faeces, cow urine, as well as oil and grease mostly in urban centers [11,12]. However, most Anopheles mosquitoes traditionally breeds in clear, clean and apparently less contaminated surroundings usually around human habitation. The factors governing the choice of water bodies by female mosquitoes to lay their eggs for breeding is still poorly understood and the knowledge of the mechanisms behind habitat selection by most species of mosquitoes is still at its infancy [13]. What is clear though is that mosquito breeding sites are located in a variety of environments including contaminated and uncontaminated environments. Thus, larval productivity, development, and survival may be attributed to degree of contamination, possible priming and selectivity for chemical tolerance and cross extended insecticide resistance [8,9,14].

Furthermore, insects, like most eukaryotes, have evolved a complex capacity to transform compounds they encounter in their environments. The development of this ability is important to their survival particularly in chemically unfriendly environments. All insects possess detoxification mechanisms, but the type, nature and capacity differs in different insect species, developmental stages, and the type of the environmental exposure [15]. Mosquitoes are of particular interest because of their role as vector of many human diseases including malaria, yellow fever, dengue fever etc. In mosquitoes, like other insect species, the challenge of responding to varieties of xenobiotic assault is compounded by the varieties of breeding ecologies and food sources upon which they rely for their life cycle. The ubiquity of mosquito breeding habitats mean that mosquitoes are found in virtually all environments from arctic to the deserts [16]. An. gambiae in particular is a highly anthropophilic malaria vector distributed widely in sub-Saharan Africa. This region constitutes 90% of the global malaria burden. Exposure of *An. gambiae* to this array of environmental xenobiotics could undoubtedly select them for tolerance and adaptive responses. Some of these responses could constitute challenges to the insecticide-based approaches to malaria management and control initiatives [17].

An elaborate three phase detoxification system is used by all animal species including *An. gambiae* to defend themselves against the toxic effects of these environmental xenobiotic substances. The three phase system metabolize the toxic substances into a less harmful one and excrete them out of the cell [18]. Among these detoxification phases, the phase I detoxification mechanism is the most elaborate; employing activities of enzymes belonging to the cytochrome P450 family. In phase II, the by-products of phase I reaction are further detoxified by means of enzymes belonging to the Glutathione S-Transferase and α & β-esterases families [19]. When organisms are exposed to environmental toxicants, a transcriptional response is activated which leads to upregulation of the genes involved in the detoxification machinery. This is called induction. Induction of detoxification enzymes in response to xenobiotic exposure has received greater attention in higher animals, because of its important implication in drug metabolism and discovery. Studies on induction of detoxification enzymes in insect vectors have tended to focus more on adaptation; how a particular strain of insect has adapted to a particular environment which could then select it for insecticide resistance [20]. However, evidence have emerged that insects like other higher animals have the ability to regulate the transcription of their detoxification genes in response to environmental xenobiotics.

Several previous studies [21,22,23,24,] have established the impact of several breeding sites ecogeographical, topographical, agricultural, and other environmental indices on Anopheles larval diversity, abundance, and dynamics, as well as breeding sites productivity. Also, induction of detoxification enzymes by various environmental xenobiotics in many species of insects has been well documented [25]. However, few published data are available on the physical and chemical nature of *An. gambiae* breeding sites and its impact on larval behavior, productivity, development, and survival. Further, *An. gambiae* have not featured prominently in studies involving induction of detoxification enzymes in response to the physical and chemical nature and characteristics of their breeding ecologies. Therefore, this study examines *An. gambiae* larval productivity and detoxification enzymes profiles in response to levels of various physico-chemical environmental factors present in their breeding sites. The aim of the study is evaluate the relationship between larval density- as a function of larval productivity-and levels of the environmental factors and to determine the inductive effect of these environmental factors on the activities of the detoxification enzyme. The environmental physicochemical factors were found to induce increased activities of the three major detoxification enzymes (P450, GST and α & β-esterases) and these increased activities were found to correlate with levels of the environmental factors across the three life stages of *An. gambiae*. Also *An. gambiae* larvae were found to produce high degree of tolerance to high levels of these environmental factors.

2. MATERIALS AND METHODS

2.1 Study Sites and Zones

The study was conducted across three different breeding sites designated as study zones A, B & C. These zones were differentiated by the type of human related activities taking place around the mosquito breeding sites i.e. A; intensive agricultural areas; B, residential areas; and C, areas where petrochemical products are sold,

processed, used and/or discharged. The breeding sites located in intensive agricultural zones and petrochemical areas consist of small puddles of stagnant water bodies. The water was found to be muddy, dirty, oily and obviously contaminated. The breeding sites in the domestic areas were larger with higher water volume and relatively clean. A total of three sites in study zone A, four in zone B and three in zone C were visited and sampled across the Nigerian states of Kano and Jigawa (Fig. 1). Kano is situated in the northwest and has a four-season climate with a typical temperature range of 11-44°C and yearly rainfall of 1000 mm. Jigawa is also situated in the northwest, and is characterized by a Sahel savannah climate with a typical temperature range of 10-42°C and a yearly rainfall of less than 800mm [26,27].

The towns and villages where the study was conducted in both Kano and Jigawa states were highlighted in green color; while the locations of the two states within the larger Nigeria map was also highlighted in colors.

2.2 Larval Sampling, Sorting, Rearing and Determination of Larval Density

Sampling of mosquito larvae from breeding sites identified in each of the three study zones was conducted at least once a week throughout the field study period (June-September, 2011). Stagnant water bodies within and/or around farmlands (including irrigated fields), residential areas and sites of petrochemical commercial activities were surveyed for the presence of mosquito larvae. Larval collections were made using 350 ml standard mosquito dipper [28]. Several dips (7-10) were made depending on the size of the breeding habitat. The dips were made from the middle of the breeding water to the surface as *An. gambiae* larvae usually dive downward when disturbed. After each larval collection, the larvae were sorted first on the basis of species into Culicines and Anophelines. The stage of the larval development, i.e., 1st, 2nd, 3rd and 4th instars was noted. All members of the Culicine species were returned back to the breeding habitat after sorting and counting while the Anopheles larvae were retained in the same breeding water after counting, in light colored plastic containers. Anopheles larvae in each dip were estimated by manual counting and overall larval densities were determined and expressed as number of larvae per liter of breeding water. Some of the sampled larvae were reared into pupae and adult.

2.3 Species Identification

An. gambiae intraspecie identification was carried out by the standard PCR procedure described by Scott [29]; courtesy of the Nigerian institute of medical research, Yaba, Lagos-Nigeria.

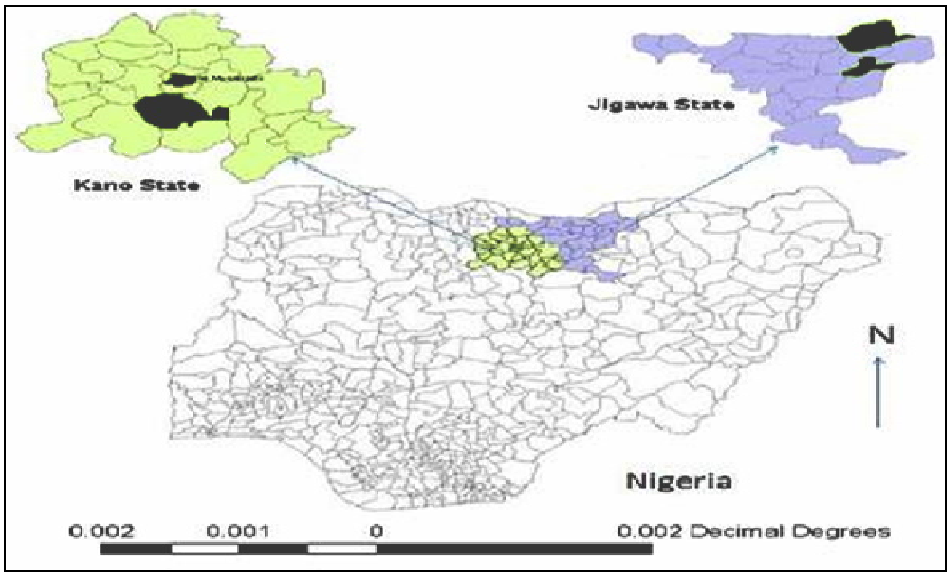

Fig. 1. Map of Nigeria projecting the locations of the field studies in Kano and Jigawa states of northern Nigeria

2.4 Water Chemistry Analysis

Conductivity, pH, temperature, and total dissolved solids were measured using combo pH/EC/TDS/temperature meter (HANNA instruments, Rhode Island, United States). Transparency (turbidity) was determined using a Secchi disc. Dissolved oxygen (DO) and biological oxygen demand (BOD) were determined using a DO meter (HACH LANGE, Colorado-united states) as described by Maiti [30]. Nitrate (NO3-), Nitrite (NO2-), Phosphate (PO32-), and Sulphate (SO42-) ions concentrations were determined by the sulphanilamide-n-(1-naphthyl)-ethylenediamine dihydrochloride (NED dihydrochloride) colorimetric, phenol disulphonic acid, stannous-chloride and turbidimetric methods, respectively [31]. Carbon content (total organic carbon) was determined using the LANGE TOC cuvette-test (Salford United Kingdom). Levels of oil and grease were determined by the liquid-liquid extraction method described by Maiti [31]. Analytical grade chemicals and reagents used were from Sigma-Aldrich (United Kingdom) and BDH chemicals (VWR international ltd. United Kingdom) unless otherwise indicated.

2.5 Detoxification Enzymes Assays

Assay of the three major detoxification enzymes, cytochrome P450 (P450), glutathione s-transferase (GST) and α & β-esterases, were carried out according to the procedures outlined by WHO [31] and by Poupardin [32]. 20 fourth instar larvae were used to prepare each homogenate.

2.5.1 P450 activity assay

Twenty (20) µl mosquito homogenate was mixed with 80 µl of potassium phosphate buffer in a microliter plate well and 200 µl of the working solution (5 ml methanol solution of 0.002 mg/ml of 3, 31, 5, 51-tetramethyl benzidine in 15 ml of 0.25M sodium acetate buffer; pH 5.0) was added. Finally, 25 µl of 3% hydrogen peroxide was added to the well. The mixture was incubated at room temperature for 2 h and the absorbance was read at 650nm using a microplate reader (Modulus Microplate reader; Turner Biosystems Sunnyvale, California, United States). Control and calibration standards (varying concentrations of standard cytochrome c) were treated similarly and all assays were performed in triplicates. P450 activity was estimated by comparing absorbance values with a standard calibration curve of absorbance for known concentrations of cytochrome c. The values are reported as equivalents units of cytochrome P450/mg protein.

2.5.2 GST assay

10 µl mosquito homogenate was mixed with 200 µl of GSH/Chlorodinitrobenzene (CDNB) working solution (125 µl of 63 mM CDNB in 2.5 ml of 10 mM GSH solution) in a microtitre plate well. The reaction was read immediately at 340 nm as a kinetic assay for 5 min. Blanks were prepared with 10 µl of the phosphate buffer mixed with 200 µl of the GSH/CDNB working solution and all the assays were performed in triplicates. The GST activity was reported as µmol CDNB conjugated/min/mgprotein, using published extinction coefficient (4.39 mM-1) corrected for the path length.

2.5.3 Esterase assay

Twenty (20) µl mosquito homogenate was mixed with 200 µl of 1-naphthyl working solution (1 ml of 30 mM 1-naphthyl acetate mixed with 99 ml of potassium phosphate buffer; ph 7.2) and 2-naphthyl acetate working solution (1 ml of 30 mm 2-naphthyl acetate mixed with 99 ml of potassium phosphate buffer; pH 7.2) in separate microtitre plate wells for α and β-esterases assay respectively and incubated for 15 minutes at room temperature. 50 µl of fast blue b stain solution was then added to the wells. A separate blank was set up for each of the two esterases containing 20 µl of potassium phosphate buffer also mixed with 200 µl of the working solutions and 50 µl of stain solution. the mixture was read at 570 nm as an end point assay using a microplate reader (Modulus microplate reader; Turner Biosystems Sunnyvale, California, United States). All the assays were performed in triplicates. Absorbance levels for each sample were compared with standard curves of absorbance for known concentrations of α-naphthol and β-naphthol to estimate the activities of α and β-esterases respectively. The results were reported as µmols of the product formed/min/mg protein.

2.6 Data Analysis

Significance in mean distribution of the environmental factors and the larval densities across the three study zones were first

investigated using mixed effect linear model with study zones as fixed factor and sites as random factor followed by Bonferoni post-hoc test for multiple comparisons. Similarly significance in mean distribution of the detoxification enzymes across the three study zone was investigated using one-way ANOVA followed by Tukey post-hoc test for multiple mean comparisons. The association or correlation between each environmental factor and the larval density as well as the detoxification enzymes activities was analyzed using bivariate linear regression with larval density and enzyme activity as the fixed factor and the environmental factors as the response variables. To assess the effect of the physicochemical environmental factors on the larval density and detoxification enzyme activities, preliminary multiple regression analysis indicated strong colinearity between model covariates. As a result of colinearity, the standard error estimates of the linear regression model get inflated and so the p-values indicating the contribution of different covariates to the model became unreliable. The colinearity problem was addressed by performing a regression in principal components, extracted from the model covariates. Factor analysis was used to extract the principal components (or principal factors) from both the environmental factors and detoxification enzymes variables, followed by a varimax rotation of the principal component axes, to allow a better alignment of the extracted components to the original environmental and enzymatic factors. Then, regression in principal components (regression between the extracted principal components from the environmental factors and the larval density) was used to investigate the effect of the environmental factors on larval density. Finally, effect of the physicochemical environmental factors on the detoxification enzymes was assessed using Redundancy analysis; involving regression between the extracted principal components of the physico-chemical environmental factors and those of the detoxification enzyme activities that were explaining 99% of the variability in both cases. All the analyses were carried out with SPSS (SPSS inc. SAS Institute) version 20.

3. RESULTS AND DISCUSSION

3.1 Specie Identification

The Anopheles mosquitoes used belong to the *An. gambiae* species sensu stricto.

3.2 Mean Distribution of *An. gambiae* Larval Density Across the Three Study Zones

The results of one way ANOVA shows that there was a significant difference (P=0.001) in mean larval distribution of *An. gambiae* across the three study zones with zone B (residential/domestic) having the highest mean larval density of 75/l (Fig. 2). The sampling sites within the intensive agricultural zone A were 2nd to those in zone B with a mean larval density of 29/l, which is about 60% less than those recorded for zone B. The sites within the petrochemical laden breeding zone C had the lowest larval densities whose mean was about 19/L, and1. 5 and 4-fold lower than those observed for zones A and B, respectively. The results of the Bonferoni post-hoc test carried out to examine the pairwise mean distribution of larval density across the three study zones showed that there were significant differences (P=0.001) in mean larval distribution between zone A & B; B & C; and A & C.

Zone A, intensive agriculture; zone B, domestic/residential; and zone C, petrochemical. Larval density (per litre of breeding water) was determined by estimating the amount of larvae in a given standard dipper. *: Significantly different from A & B. All the larvae used were at fourth instar stage. The error bars represent standard deviation. Part of legend for Fig. 2. Should be at the bottom of the figure.

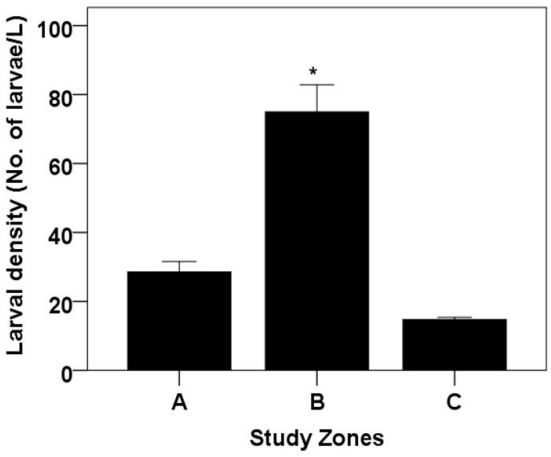

Fig. 2. Distribution of mean larval density across three different *An. gambiae* breeding sites in northern Nigeria

3.3 Mean Distribution of Physico-chemical Environmental Factors Across Three Study Zones

Results of the mixed effect linear model showed that the mean distribution of pH, temperature, conductivity DO, BOD, and transparency were not statistically significant (Fig. 3) across the three study zones. Likewise, the differences in mean distribution of total dissolved solids, sulphates, phosphates, nitrites, nitrates, carbon content and oil and grease across the three study zones were significant (P=0.001) (Fig. 3). The Bonferoni post-hoc pairwise comparison tests showed that comparing mean distribution between zone A & B; A & C and B & C for all the physical environmental factors were not significant. For instance, for pH, the zone-wise comparisons between zone A against B, A against C and B against C was statistically not significant. For dissolved oxygen (DO & BOD), there were also no statistically significant differences in mean zone-wise comparisons between zone A against B, A against C and B against C. Lastly, same zone-wise comparisons for temperature, conductivity, and transparency were also not statistically significant. However, the zone-wise comparisons for TDS and the environmental chemical factors (sulphates, phosphates, nitrites, nitrates, carbon content and oil and grease) were all statistically significant (Fig. 4) (P=0.001).

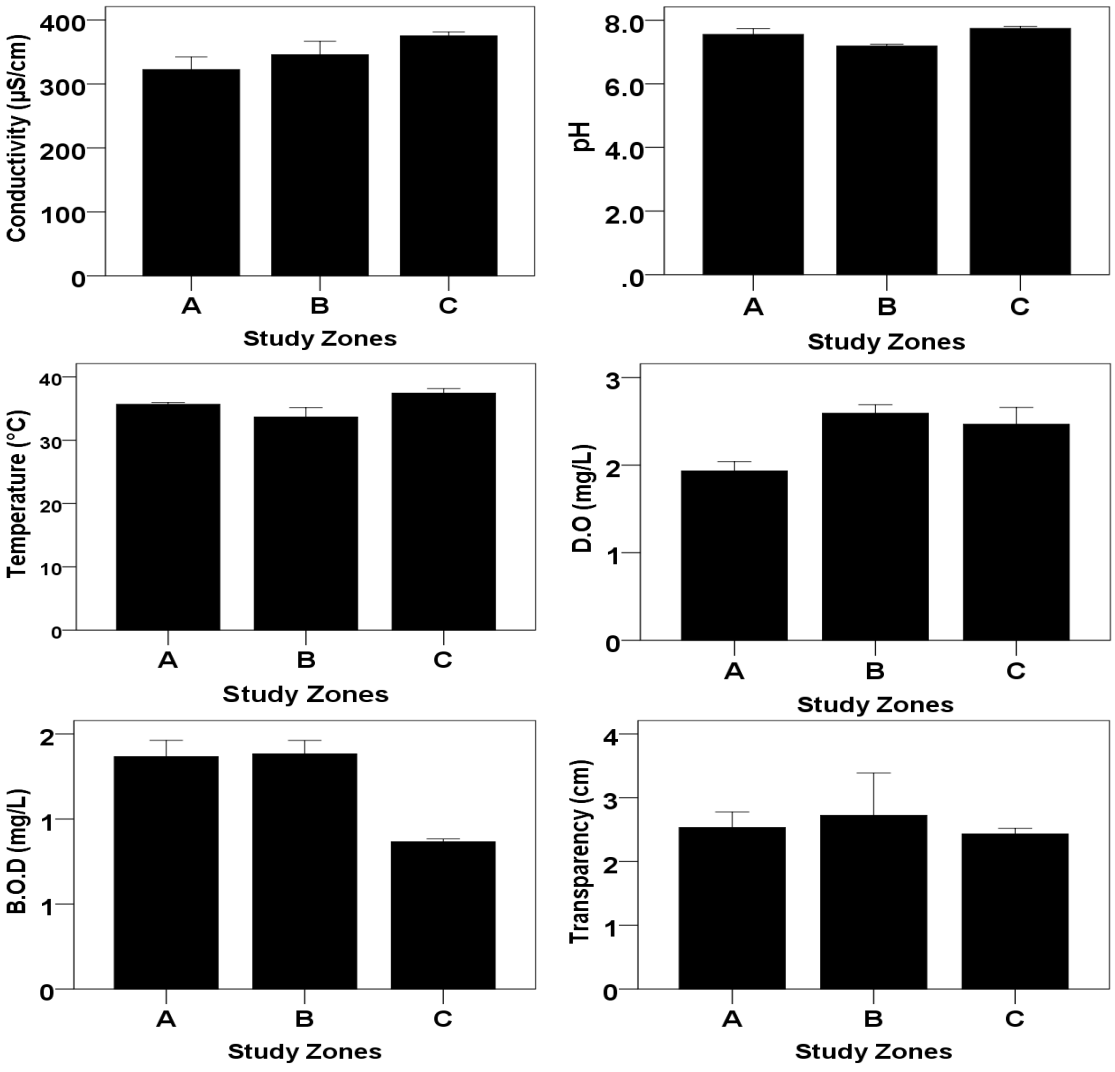

Fig. 3. Mean distributions of environmental physical factors across three different *anopheles gambiae* breeding sites in northern Nigeria

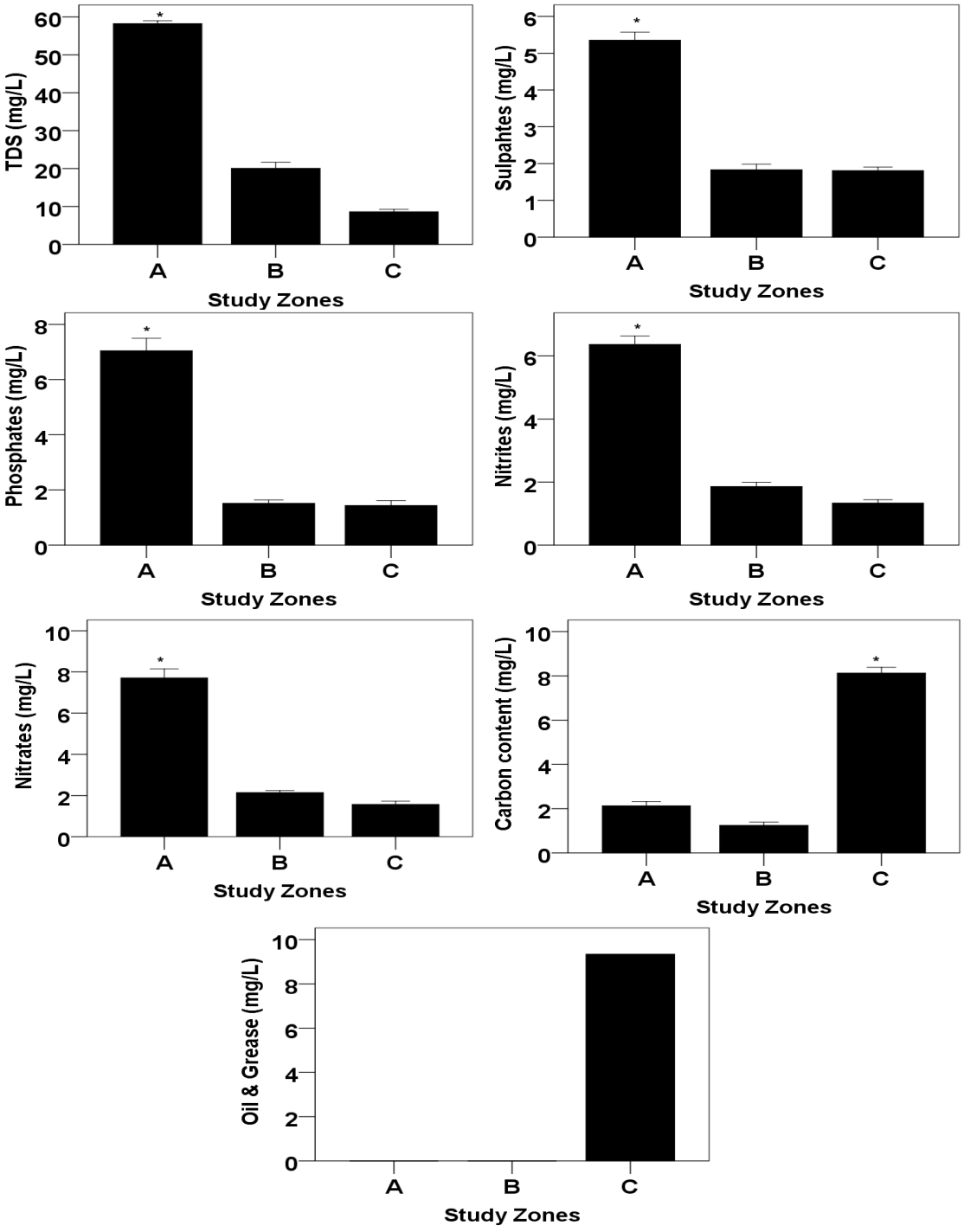

Fig. 4. Mean distributions of chemical environmental factors across three different *Anopheles gambiae* breeding sites in northern Nigeria

3.4 Mean Distribution of the Detoxification Enzymes Across the Three Study Zones

The results of the mixed effect linear model showed that the differences in mean distribution of *An. gambiae* larval P450 activities across the three study zones was statistically significant (*P*=0.001) with highest mean distribution recorded in study zone C (petrochemical laden). The mean larval P450 activities of zone A and B were 1.7 and 4.3-fold lower than that of zone C. (Fig. 5). Additionally, Bonferoni post-hoc pair-wise comparison test showed significant differences in mean larval P450 activities between zone A and B (*P*=0.001) and B & C (*P*=0.001). The difference between A and C was moderately significant (P=0.160). In contrast, *An. gambiae* larvae from study zone A (intensive agricultural sites) recorded the highest mean GST and α & β-esterases activities compared to the other two zones. The differences in mean distribution of these two detoxification enzymes between zone A & B and A & C were significant (*P*=0.001) while differences between zone B & C was not (Fig. 5).

3.5 Association between Each Physico-chemical Environmental Factors, Larval Density and Detoxification Enzyme Activities

Preliminary investigation based on bivariate linear regression analysis between larval density and each physical environmental factor shows that both pH and temperature were negatively correlated (P<0.05,) with larval density, while transparency and BOD were positively associated (P<0.05) with larval density. The physical environmental parameters that appeared not to influence larval density were conductivity and dissolved oxygen. Furthermore, selected chemical environmental factors (sulphates, phosphates, nitrites, nitrates,) produced a moderate negative correlation (P<0.05) with *An. gambiae* larval density while carbon content and oil and grease produced significant associations (P<0.05) with *An. gambiae* larval density. This suggests that as the levels of these chemical environmental parameters increase, the density of *An. gambiae* larvae decreases. As was observed with dissolved oxygen and conductivity, the total dissolved solids had no significant association with larval density.

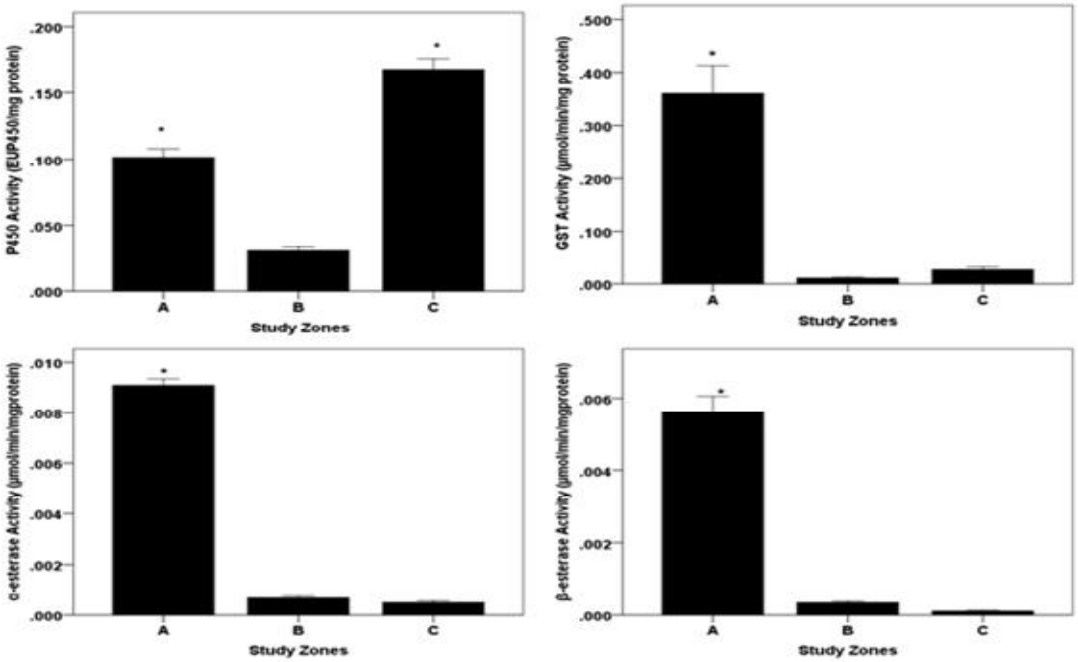

Fig. 5. Mean distribution of the three major detoxification enzymes (P450, GST and α & β-esterases) at the larval stage of Anopheles gambiae sampled from three different breeding sites in Northern Nigeria

Likewise, bivariate linear regression analysis between each physico-chemical environmental factors and the detoxification enzyme activities showed that pH and temperature were statistically positively associated (P 0.001 and 0 .001) respectively with larval P450 activities while BOD showed significant (P=0.001) negative correlation. There were also no significant associations between Conductivity, DO, transparency and larval P450 activity (P<0.05). Furthermore, the chemical environmental factors; TDS, sulphates, phosphates, nitrites, and nitrates were not significantly associated respectively, with larval P450 activity while carbon content and oil and grease were significantly positively correlated (P=0.001) with larval P450 activity. This means increase in the levels of carbon content and oil and grease produced increased larval P450 activity. For GST and α & β-esterase activities, pH, conductivity, and DO were all significantly associated (P=0.001), while temperature, BOD and transparency respectively, were not. In contrast to P450, the chemical environmental factors; TDS, sulphates, phosphates, nitrites and nitrates showed very strong positive correlation (P= 0.001) with larval GSTα & β-esterase activities while carbon content and oil and grease displayed weak negative associations respectively with GST α & β-esterase activities at this life stage. Similar observation were also recorded for the pupae and adult samples.

3.6 Combined Effect of the Physico-chemical Environmental Factors on Larval Density and Detoxification Enzymes Activities

In order to deduce a statistical model showing a combination of the physico-chemical environmental factors that produce the most combined significant effect on larval density and detoxification enzymes activities, factor analysis was carried out on both the physico-chemical and detoxification enzymes variables followed by redundancy analysis between the extracted principal components of the detoxification enzymes and those of the physico-chemical environmental variables. Regression in principal component (between larval density and the extracted principal components of the physico-chemical environmental factors) was carried out to establish the effect of the physico-chemical environmental factors on larval density. Preliminary classical multivariate regression between the environmental variables and the detoxification enzymes failed to produce a reliable model estimates due to strong colinearity among the physico-chemical variables as well as among the detoxification enzymes. Therefore factor analysis was employed to extract components from both the physico-chemical variables and the detoxification enzymes.

The SPSS results of the factor analysis on the physico-chemical environmental factors showed that the first eight principal components explained 99% of the variability in the thirteen environmental variables (Fig. 6.), and therefore, only these first eight components were retained in the regression analysis. According to the factor loadings (Fig. 6), the first principal component (PC1) correlated strongly with TDS, sulphates, phosphates, nitrites, and nitrates; PC2 correlated strongly with carbon content and oil and grease, PC3 was explained by conductivity, PC4 by temperature, PC5 correlated with transparency, PC6 was associated with DO, PC7 is explained by pH while PC8 is correlated with BOD. Thus, the first component (PC1) represents contamination from pesticides and chemical fertilizer application (fertilizer and pesticides contaminants), PC2 represents contamination from the sale, use, processing, and/or discharge of petrochemical/hydrocarbon products (petrochemical contamination) while PC3-8 represent the physical environmental variables.

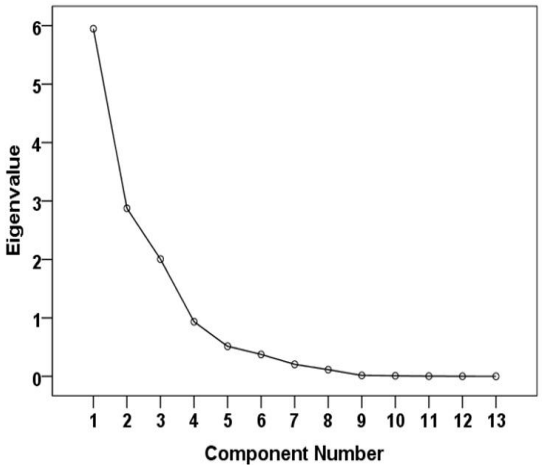

Fig. 6. Scree plot showing the eigenvalues of the principal components from the factor analysis of the physico-chemical environmental variables

The results of the regression in principal components (Table 1) showed that only six out of the eight principal components included in the analysis had a significant effect on larval density. The components producing a significant effect on *An. gambiae* larval density in Nigeria comprise pesticides and fertilizer contaminants (PC1), petrochemical/hydrocarbon contaminants (PC2) and the physical environmental variables; conductivity (PC3), temperature (PC4), transparency (PC5), and pH (PC7). In turn, neither DO (PC6) nor BOD (PC8) was found to produce any significant effect on larval density (Table 1).

Furthermore, the result of the factor analysis carried out on the detoxification enzymes produced three extracted principal components (PC) which explained 99% of the variability in the data. According to the factor loading (Fig. 7) the first principal component (PC 1) correlates strongly with GST and α and β-esterase activities, PC 2 correlated with the P450 enzyme activities whereas PC 3 was associated with GST alone. Therefore, according to these results, these three principal components explained more than 99% of all the variability in the detoxification enzymes irrespective of life stage.

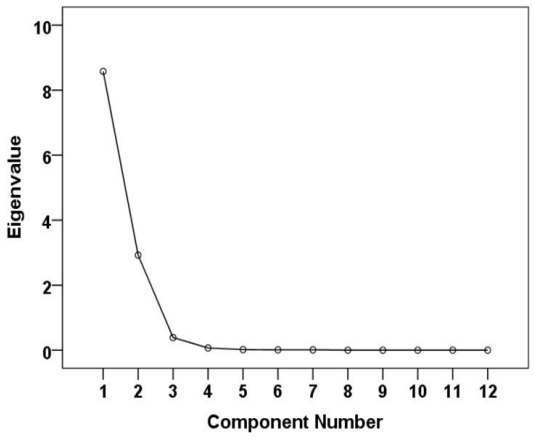

Fig. 7. Scree plot of the extracted components from factor analysis of the detoxification enzyme variables. Components 1-3 explained 99% of the variability in the data

The result of redundancy analysis (Table 2) between the principal components extracted from the physico-chemical environmental variables (i.e. PC1-8; Fig. 6) and GST and α and β-esterases activities (i.e. PC 1&3 of the detoxification enzyme variables; Fig. 7) showed that all pesticide and fertilizer contaminants, the petrochemical/hydrocarbon contaminants and only the physical environmental factors; pH, conductivity, transparency, DO and BOD produced the most combined significant effect on the activities of these two enzymes (i.e. GST and α and β-esterases).

Lastly, the result of the redundancy analysis (Table 3) between the extracted components of the environmental physico-chemical factors and P450 activities (i.e. PC 2 of the detoxification enzyme variables; Fig. 7) showed that, unlike GST and α & β-esterase enzymes, all the eight extracted components of the physico-chemical environmental variables (Fig. 6) produced a combined effect on P450 activities. Thus pesticide and fertilizer contaminants, petrochemical contaminants and the physical environmental factors; pH, temperature, conductivity, transparency, DO and BOD all produced a combined effect on the activities of P450 at the larval stage of *An. gambiae* from northern Nigeria.

4. DISCUSSION

This study demonstrated that the density of *An. gambiae* larvae were significantly influenced by several physicochemical environmental factors that are associated with mosquito breeding sites. These environmental factors were functions of the human related activities going on around these breeding site. The levels of TDS, sulphates, phosphates, nitrites and nitrates were significantly higher in sampling sites within study zone A. This could be explained by the use of nitrate and phosphates-base fertilizers as well as agro-allied pesticides in farmlands located around these mosquito breeding sites. The levels of carbon content and oil and grease were highest in breeding sites located in study zone C, which is consistent with the major types of human activities taking place. There is widespread sale, processing, use and discharge of petroleum products in this zone which appeared to be exacerbated by fuel (petrol or premium motor spirit) vending, a common practice in northern Nigeria due to constant chronic fuel shortages. In addition, kerosene is a major domestic fuel in Nigeria and is normally sold by small retailers who are usually located within and/or around human habitats. Activities of automobile and motorcycle mechanics, which are often located around human habitation, are also major additional sources of discharged spent fuel

Table 1. Environmental physicochemical factors or components with combined effect on *An. gambiae* larval density

Parameter	Coefficient	Std. error	Wald chi-square	df	sig
Intercept	42.999	0.3749	13156.397	1	<0.001
PC1	-13.2	0.3813	1198.567	1	<0.001
PC2	-22.414	0.3813	3455.792	1	<0.001
PC3	-10.962	0.3813	826.525	1	<0.001
PC4	-5.152	0.3813	182.503	1	<0.001
PC5	12.301	0.3813	1040.873	1	<0.001
PC6	-0.136	0.3813	0.126	1	0.722
PC7	-7.684	0.3813	406.144	1	<0.001
PC8	0.051	0.3813	0.18	1	0.893

df: degree of freedom; Sig; Significance

and lubricants into surrounding water bodies and mosquito breeding sites. The density of *An. gambiae* larvae was negatively associated with the levels of all the chemical parameters analyzed in this study i.e. sulphates, phosphates, nitrites, nitrates, carbon content and oil and grease, all of which were also selected in model of environmental factors (Table 1) producing a combined effect on larval density. This means that increase in the levels of these chemical species lead to decrease in the density of *An. gambiae* larvae. The presence of *An. gambiae* larvae in breeding sites containing high levels of chemical species suggest a gradual potential emergence of tolerance of *An. gambiae* larvae to these environmental chemical factors, especially that the relative lower larval densities recorded in zones A (agricultural) and C (petrochemical) in comparison with zone B (domestic/residential) are equally high when compared to other studies [33, 34, 35, 36, 8, 14, 9, 37, 10]. The results and analysis of the detoxification enzymes activities (Fig. 5, Tables 2 & 3) recorded in this study suggests that the observed tolerance of *An. gambiae* to these environmental physico-chemical factors could be linked to or explained by the activities of these detoxification enzymes. In general, induction of detoxification enzymes in response to several xenobiotic exposures in many insect species has been well documented [19,25,38,39]. However, few studies have, to my knowledge, demonstrated the inductive capacity of the environmental chemical species considered in this study on the activities of detoxification enzymes in *An. gambiae* in Northern Nigeria, despite considerable evidence which indicate that Anopheles mosquitoes thrives in breeding sites where they could be exposed to these environmental chemicals.

The aim of this present study is to establish the potentiality of different physico-chemical environmental factors as driving a selection pressure for the emergence and development of insecticides resistance in *An. gambiae*. This is because of the similarity in structures, functions and activity relationships between these environmental factors and several synthetic insecticides used in mosquito control. The hypothesis here is that prior exposure of products containing these chemical species present in *An. gambiae* breeding sites, could exerts a selection pressure that could drive an intrinsic and acquired capacity in *An. gambiae* towards tolerance to several types of insecticides used for its control. One of the major mechanisms for the development of insecticide resistance in mosquitoes is detoxification enzymes mechanism. This involve increase in the activities of detoxification enzymes (P450, GST α and β-esterases) which lead to rapid metabolism of the insecticides before it reaches its sites of action [25,40]. Therefore, exposure of *An. gambaie* to different environmental chemical compounds present in its breeding sites, which could induce increase in activities of these enzymes could potentially produce intrinsic and acquired tolerance to insecticides in mosquitoes emerging from such breeding sites, especially if these chemical compounds possess similar structures and activity relationship with the various insecticides used in mosquito control.

A comparative analysis between activities of the detoxification enzymes recorded in this study and those of *An. gambiea* displaying metabolic resistance to DDT and pyrethroids insecticides in Nigeria's West African neighbors; Burkina Faso and Cameroon was carried out. Previous studies [41,42,43] in Burkina Faso and Cameroon had demonstrated DDT and pyrethroids metabolic resistance in *An. gambiae.* In these studies, activities of the three major detoxification enzymes were implicated, among

Table 2. Environmental physicochemical factors or components with combined effect on GST and α & β-esterases at the larval stage of *An. gambiae*

Parameter	Coefficient	Std. error	Wald chi-square	df	sig
Intercept	8.072E-016	0.0194	0.000	1	1.000
PC1	0.955	0.0198	2334.535	1	<0.001
PC2	-0.109	0.0198	30.603	1	<0.001
PC3	-0.106	0.0198	28.561	1	<0.001
PC4	-0.010	0.0198	0.256	1	0.607
PC5	-0.070	0.0198	12.720	1	<0.001
PC6	-0.196	0.0198	98.394	1	<0.001
PC7	-0.006	0.0198	0.081	1	0.776
PC8	-0.099	0.0198	25.297	1	<0.001

Table 3. Environmental physicochemical factors or components with combined effect on P450 at the larval stage of *An. gambiae*

Parameter	Coefficient	Std. error	Wald chi-square	df	sig
Intercept	7.181E-016	0.0073	0.000	1	1.000
PC1	0.143	0.0074	373.714	1	<0.001
PC2	0.940	0.0074	16178.348	1	<0.001
PC3	0.038	0.0074	25.838	1	<0.001
PC4	0.233	0.0074	993.202	1	<0.001
PC5	-0.093	0.0074	159.984	1	<0.001
PC6	0.138	0.0074	350.375	1	<0.001
PC7	0.076	0.0074	107.203	1	<0.001
PC8	0.075	0.0074	103.993	1	<0.001

other mechanism, as conferring resistance in their resistant strain of *An. gambiae* in comparism to the Kisumu strain which was used as reference susceptible standards in all of these studies. Comparing the detoxification enzyme activities of the resistant and susceptible reference Kisumu strains reported in these previous studies with the activities recorded in this present study showed that P450 and GST activities favorably compared, and even in many cases, higher than those reported in Burkina Faso and Cameroon resistant strains [41,42,43]. However, α-esterase activities recorded in this present study was lower than those from these previous studies. Interestingly, most of the lowest P450 and GST activities recorded in this study were higher than those of the Kisumu susceptible reference standards used and reported in the Burkina Faso and Cameroon studies. While this comparative analysis was not intended to indicate that the *An. gambiae* samples in this study were also resistant to these insecticides, the result nevertheless serve to establish comparism with strains of *An. gambiae* confirmed to be displaying metabolic resistance to various insecticides through the activities of these detoxification enzymes. However, the results suggest that the population of mosquitoes in some of these breeding sites studied in Northern Nigeria may have developed or are selectively being primed to develop resistance to insecticides.

This study thus revealed that *An. gambiae* emerging from breeding sites located in study zone A and C could be selected for potential tolerance to insecticides, especially those having similar structures and activity relationship to the environmental chemical compounds present in high levels in these breeding sites. Several previous studies [44,45,46] have demonstrated the contribution of prior exposure to various environmental xenobiotics to the development of insecticides resistance by several insect species. In addition, other studies [32,41,47,48] have also established a correlation between increase in tolerance to insecticides in many insects and induction of detoxification enzymes as a result of prior exposure to environmental xenobiotics. While many of these previous studies established relationship between exposures to some environmental xenobiotics and incidence of insecticides resistance in various mosquito species, this present study was conducted at the level of pre-insecticide exposure to implicate some broad-base human activities, such as

those described in this study, as potentially driving intrinsic and acquired tolerance to insecticides in *An. gambiae* in northern Nigeria. Moreover, *An. gambiae* has not featured prominently in many of these previous studies and none of these studies have to my knowledge been carried out in northern Nigeria. Thus, this study became necessary in view of the fact *that An. gambaie* is the major malaria vector in Nigeria [49] and Nigeria accounts for the highest malaria deaths in Sub-Saharan Africa [1].

Findings from this study were also consistent with observations from several previous studies which implicated agricultural practices as a selection factor in the development and emergence of insecticides resistance in various insect species from many parts of the world. For instance, Georghiou [50] demonstrated organophosphate resistance in *An. albimamus* following intensive treatment of cotton pest with pesticides in El-Salvador. The resurgence of malaria in India and Central America was linked to intensive agricultural production employing intensive use of agricultural pesticides [51]. Brogdon [52] demonstrated elevated levels of acetylcholinesterase activity in *An. albimamus* in intensively managed agricultural areas in Guatemala. Furthermore, a comparative analysis involving two malaria vectors; *An. nigerrimus* and *An. culcifaciens* was carried in Sri Lanka. The former breeds in intensive agricultural areas while the latter breeds in non-agricultural areas. The results of the analysis showed that *An. nigerrimus* was resistant to organophosphate and carbamates at both larval and adult stages while *An. culcifaciens* was not. This established the role of agriculture as source of selection pressure for development of resistance in *An. nigerrimus* [53]. These and other similar studies have established the impact of agricultural practices in the emergence and development of insecticides resistance. Majority of these studies focused primarily on the role of the use of agricultural pesticides as selection factor in the development of resistance to public health insecticides. But other agrochemicals other than pesticides, such as fertilizer studied here, could also play an important role. In addition, most of these previous studies were conducted at the level of post-insecticides exposure. However, similar studies carried out at the level of pre-insecticides exposure are necessary in order to evaluate the extent and nature of the role of various agricultural practices as selection pressure for the development and emergence of insecticides resistance in public health vectors. Thus, this present study, which to my knowledge is the first of its kind in Northern Nigeria, aim to bridge this gap by assessing the importance of two major agricultural practices; pesticide and fertilizer application, as potential sources of selection pressure for the development and emergence of insecticides resistance in *An. gambiae* in northern Nigeria.

This study also revealed that *An. gambiae* larvae thrive in breeding sites laden with petrochemical/hydrocarbon products. Larval prospecting and collection was conducted in these habitats and large amount of *An. gambiae* larvae were collected from these breeding sites. High degree of larval tolerance to these petrochemical products was established and biochemical enzyme analysis of *An. gambiae* mosquito collected from these breeding sites revealed that the activities of P450 monooxygenase enzyme was significantly higher in these petrochemical laden breeding zone relative to the other two studied zones (zone A and B). Statistical analysis also showed that carbon content and oil and grease; levels of which were significantly higher in breeding sites located in this zone compared to intensive agriculture and residential zones were strongly positively associated with P450 activities. The model deduced from both regression in principal component and redundancy analysis for the combined effect of physico-chemical factors larval density on P450 activities respectively, also prominently selected carbon content and oil and grease as producing the most significant influence on larval density as well as the activities of P450 across the three life stages of *An. gambaie*. Thus, these observations showed that petrochemicals or hydrocarbon products could induce significant P450 activities which could leads to high degree of tolerance to these compounds by *An. gambiae*. Although, this study could not conduct elaborate hydrocarbon profile of these breeding sites, therefore, it is not clear which specific hydrocarbon specie is responsible for the observed inductive effect.

The effect of the presence of petrochemical/hydrocarbon products on the growth, survival and biochemical behavior of *An. gambiae* has not, to my knowledge, been largely investigated, despite an age long tradition of applying these products to mosquito breeding waters to control mosquito larvae [54,55]. Findings from previous studies [56,57,58,59] have however demonstrated several effects of petrochemical products on many other aquatic

organisms in Nigeria. The induction of cytochrome P450 systems in response to exposure of insects to petrochemical products has not been largely investigated. However, since this enzyme system together with the other detoxification machinery are also conserved in insects [60], inferences from findings in other organisms can be used to explain the observations made in this present study.

5. CONCLUSION

This study has demonstrated the significance of the physico-chemical environmental factors present in mosquito breeding sites, not only on the density of *An. gambiae*, but also on their detoxification enzymes machinery. Significant associations were established between several physico-chemical environmental factors and larval density as well as activities of the three major detoxification enzymes (P450s, GSTs and carboxylesterases) in *An. gambiae.* The levels and characteristics of these environmental factors were related to the various human activities taking place around the mosquito breeding sites. Analyses of the significance of these findings as well as observations and inferences from previous studies have demonstrated the impact this study could produce on the contemporary integrated vector control approach to malaria management. For example, findings in this study should be taken into cognizance when formulating control measures such as insecticides selection, environmental management practices, integrated vector control etc.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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