Spectrum of metabolic-based resistance to DDT and pyrethroids in *Anopheles gambiae* s.l. populations from Cameroon

Josiane Etang[1,2]✉, Lucien Manga[3], Jean-Claude Toto[2], Pierre Guillet[4], Etienne Fondjo[5], and Fabrice Chandre[6]

[1]*Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale, BP 288, Yaoundé, Cameroon*
[2]*Institute of Medical Research and Studies of Medicinal Plants, P.O.Box 6163, Yaoundé, Cameroon*
[3]*World Health Organization, Regional Office for Africa, P.O. Box 6, Brazzaville, Congo*
[4]*World Health Organization, Geneva, Switzerland*
[5]*National Malaria Control Program, Ministry of Public Health, P.O. Box 14386, Yaoundé, Cameroon*
[6]*Centre de Recherches Entomologiques de Cotonou (CREC), 06 B. P. 2064, Cotonou, Bénin*

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ABSTRACT: Some populations of *Anopheles gambiae* s.l. from Cameroon were reported to develop resistance to DDT or pyrethroids but were free of the kdr mutation "Leucine-Phenylalanine" (Leu-Phe). This study reports on the metabolic activity of non-specific esterases (NSEs), mixed function oxidases (MFOs), and glutathione S-transferases (GSTs), three enzyme systems commonly involved in insecticide resistance. Biochemical assays were performed in DDT or pyrethroid-resistant populations of *An. gambiae* s.l. from Douala, Mbalmayo, Pitoa, and Simatou neighborhoods. Enzyme activity was compared to the Kisumu-susceptible reference strain using the Mann-Whitney test. Most of the tested samples had elevated NSE activity ($P<0.02$). The Douala sample evenly displayed elevated GST activity ($P<0.001$), while high MFO level was recorded in the Pitoa sample ($P<0.001$). MFO or GST levels were sometimes lower or similar to that of the Kisumu strain. These results suggest metabolic detoxification is a major DDT or pyrethroid resistance mechanism and emphasize the need for further investigations on *An. gambiae* s.l. resistance mechanisms in Cameroon. ***Journal of Vector Ecology* 32 (1): 123-133. 2007.**

Keyword Index: Metabolic-based resistance, insecticides, *Anopheles gambiae*, Cameroon.

INTRODUCTION

The emergence of vector resistance to insecticides is considered one of the main obstacles in the struggle against malaria. Numerous vector species are reported to have developed resistance to pyrethroids (Elissa et al. 1993, Vulule et al. 1994, Chandre et al. 1999a, Hargreaves et al. 2000, Hemingway and Ranson 2000), while these insecticides are strongly advocated for treatment of material used in public health (Lengeler et al. 1996, WHO 1999, Zaim et al. 2000). Resistance may occur by different physiological mechanisms including metabolic detoxification through increased enzyme activities (monooxygenases, esterases, or glutathione S-transferases) (Scott 1996, Hemingway et al. 1998), target site insensitivity due to mutations such as *kdr*, or by both mechanisms. The *kdr* leucine-to-phenylalanine (Leu-Phe) or leucine-to-serine (Leu-Ser) mutations (Martinez-Torres et al. 1998, Ranson et al. 2000) are the most well-characterized DDT and pyrethroid resistance mechanisms in *An. gambiae* s.l. The *kdr* leu-phe mutation has been extensively documented from West African populations of *An. gambiae* (Chandre et al. 1999a, Diabate et al. 2004, Awolola et al. 2005, Reimer et al. 2005), while little is known about the level of metabolic detoxification involvement in the resistance observed in this sub-region of Africa.

Elevation of one or more broad substrate spectrum esterases is another common mechanism of insecticide resistance. Most pyrethroid compounds, including deltamethrin, contain an ester linkage that is susceptible to hydrolysis by esterase (Oppenoorth 1985). The use of organophosphate and carbamate compounds may increase esterase production that confers cross resistance to pyrethroids (Brogdon and Barber 1990). Associated elevated esterase levels have been documented in many pyrethroid-resistant insects (Riskalla 1983, Beach et al. 1989, Rodriguez et al. 1997), including some *An. gambiae* populations from East Africa (Vulule et al. 1999).

Activation of mixed function oxidases (MFOs) has also been associated with pyrethroid resistance in mosquitoes (Hemingway and Ranson 2000, Brooke et al. 2001). MFOs are a cascade of enzymes, with the rate-limiting enzyme usually being cytochrome P450 (Nelson et al. 1996). Alterations in this rate-limiting enzyme can dictate the level of resistance to pyrethroids, organophosphates, and carbamate insecticides. Furthermore, GSTs have been reported to play a significant role in detoxification and insect resistance to DDT (Ranson et al. 1997, Prapanthadara et al. 1998), including *An. gambiae* in Africa (Ranson et al. 2001). However, these enzymes appeared as a defense against pyrethroids in certain insects (Kostaropoulos et al. 2001). They were found to play a minor role in *An. funestus*

resistance to pyrethroids (Brooke et al. 2001) and were not associated with pyrethroid/DDT resistance in *An. minimus* from Thailand (Chareonviriyaphap et al. 2003).

For the purpose of resistance management, evaluation of insecticide treated bednet (ITNs) efficacy was carried out in an area of pyrethroid-resistant *An. gambiae* populations with high *kdr* gene frequency, and ITNs were found to still be effective (N'Guessan et al. 2001). Furthermore, new insecticide applications like "two-in-one" provided promising results in controlling highly resistant vector populations of *An. gambiae* (N'Guessan et al. 2001, Guillet et al. 2001). Rotation of treatments in residual spraying was also reported to decrease the level of metabolic-based resistance in *An. albimanus* (Hemingway et al. 2002). However, given the events of cross-resistance and multi-resistance (Brogdon et al. 1999, Chandre et al. 1999b), the concept of resistance management is becoming more and more complex, especially in areas where the same or related chemicals are used extensively against both mosquitoes and agricultural pests. As much as possible, vector resistance monitoring should be combined with identification of the involved mechanism in order to follow up the evolution of resistance genes or to detect new resistance mechanisms.

Accordingly, we carried out biochemical assays in four populations of the *An. gambiae* complex from Cameroon previously found resistant to DDT or pyrethroids but negative for the *kdr* diagnostic test (Etang et al. 2003). The objective was to monitor the activity of esterases, oxidases, and gluthatione S-transferases, three enzyme systems commonly involved in insecticide resistance.

MATERIALS AND METHODS

Study sites and mosquito samples

The study was undertaken between October 1999 and August 2001. Study sites are indicated in Figure 1. Mosquitoes were collected from Douala (4°03'N, 9°43'E), Mbalmayo (3°31'N, 11°30'E), Pitoa (9°24'N, 13°31'E), and Simatou (10°52', 15°00'E), located respectively in coastal equatorial, guinean equatorial, tropical humid, and sahelian areas in Cameroon. Details on their climatic descriptions are given elsewhere (Etang et al. 2003).

Anopheline larvae were collected from breeding sites and reared in the insectary. They were fed fish food (TetraMikromin®). Emerged adults were fed with a 10% glucose solution. The biological materials used for biochemical assays were two- to four-day-old unfed females of *An. gambiae* s.l. that emerged from these larval collections and were used as controls (without contact with insecticides) during WHO susceptibility tests. The Kisumu-susceptible reference strain of *An. gambiae* s.s. reared in our laboratory also served as a control.

Biochemical analysis

Mosquitoes were selected from cages, immobilized by brief exposure to freezing temperatures and kept at –80° C until the date of analysis (less than a month). Each insect was ground in 200 µl distilled water and the homogenate was centrifuged at 1,4000 rpm. Two 20 µl replicates of the supernatant were transferred to two adjacent wells of a one microtiter plate for MFO assay. Non-specific esterases (NSEs), gluthatione S-transferases (GSTs), and protein assays were performed with two 10 µl replicates of supernatant.

Biochemical analyses were performed with two replicates of the homogenate from each individual mosquito. The two replicates were put in two adjacent wells of the same microplate. The number of microplates used for each population was at least one (23 individuals in two replicates) and 1/3 (seven individuals in two replicates + one blank (distilled water) in two replicates), for a total of 30 specimens. This sample distribution allowed us to analyze the two replicates from each mosquito in the same microplate at the same time, for each of the three enzyme systems analyzed.

Non-specific esterase activity was measured using the two substrates, α-naphtol acetate (αNa) and p-nitrophenyl acetate (*p*-NPA). For the αNa assay, 90 µl phosphate buffer (PBS pH 6.5) and 100 µl 0.6 M αNa were added to 10 µl of microfuge supernatant. After 30 min incubation, 100 µl of Fast Garnett BC solution (8 g Fast Garnett Salt + 10 ml distilled water) was added to stop the reaction. The concentration of the final product was determined at 550 nm as an endpoint calculated from a standard curve of α-naphtol. For the *p*-NPA assay, 200 µl of *p*-NPA working solution (20 ml 0.05M phosphate buffer pH 7.4 + 100 ml 0.2 M *p*-NPA) were added to 10 µl of microfuge supernatant. After 3 min incubation, the plate was read at 405 nm, every 20 s, for 2 min. The rate of formation of *para*-nitrophenol was determined kinetically and converted into absolute units based on the product molecular extinction coefficient (13.3 mM cm^{-1}).

The assay used for MFO detects the elevation in the amount of heme, which is then converted into equivalent units (EU) of cytochrome P450. Cytochrome P_{450} was titrated using the heme-peroxidase assay according to Brogdon et al. (1998). Eighty µl of 0.625 M potassium phosphate buffer (pH 7.2) were added to 20 µl microfuged supernatant and 200 µl tetramethyl benzidine solution (0.011 g 3,3',5,5' Tetramethyl Benzidine, i.e. TMBZ + 5 ml methanol + 15 ml sodium acetate buffer 0.25 M pH 5.0). Twenty-five µl of 3% hydrogen peroxide were added and the mixture was incubated for 30 min at room temperature. Absorbance was read at 630 nm and values calculated from a standard curve of cytochrome C.

Glutathione S-transferase assay was measured with a 200 µl GSH/CDNB working solution (100 µl of 0.060 g reduced glutathione prepared in 10 ml 0.1M sodium phosphate buffer pH 6.5 + 100 µl of 0.013g 63 mM 1-chloro-2, 4-dinitrobenzene, diluted in 1 ml methanol and 10 ml 0.1 M phosphate buffer at pH 6.5) added to each replicate of mosquito homogenate. The absorbance was read at 340 nm every 30 s for 5 min. The rate of formation of CDNB conjugated was determined kinetically using a molecular extinction coefficient (9.6 mM cm^{-1}).

Total protein content in 10 µl aliquots of microfuge

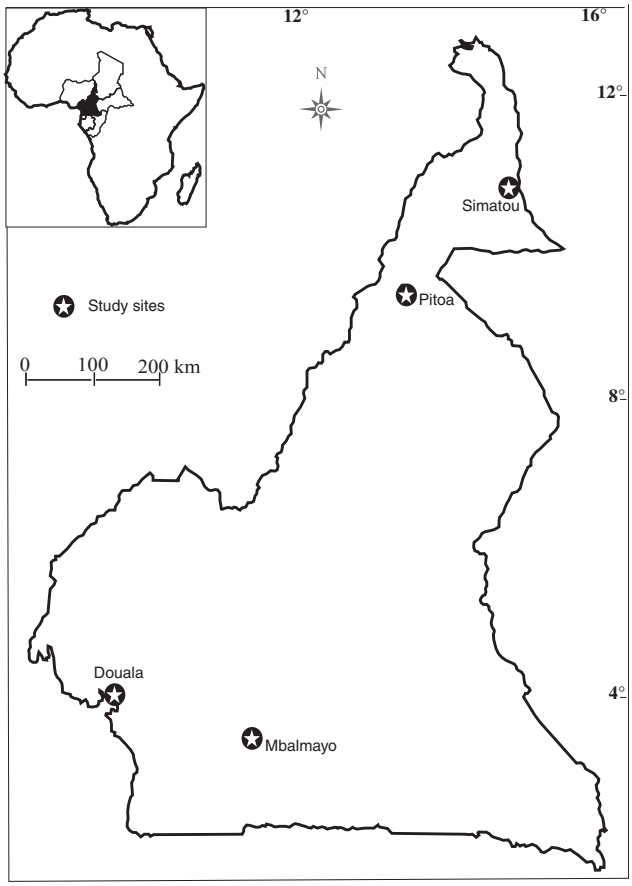

Figure 1. Map of Cameroon showing study sites.

supernatant was measured using the Bradford assay (Bradford 1976), in order to report enzyme activity to protein value in each sample. Two-hundred-ninety µl solution of Coomassie Plus Protein Assay Reagent were added to each replicate and the mixture was incubated at room temperature for 5 min. The end point absorbance was read at 590 nm. Protein values were calculated using a standard curve of absorbance of bovine serum albumin.

Data analysis

Mean absorbance values of replicate wells for each tested mosquito were converted into enzyme activity and divided by the protein values. Results were then presented graphically according to the distribution of activity in each population as the frequency of individuals observed in each class of activity. The mean activity for each sample was calculated, then the distribution was compared to that of the Kisumu-susceptible strain with the Mann-Whitney test. Levels of statistic significance were determined at $P<0.05$.

RESULTS

For each of the three enzyme systems, we analyzed 30-60 individuals. Mean activities are given in Table 1.

Non-specific esterases

The profiles of NSE activity with the substrates αNa and *p*-NPA are given in Figures 2 and 3, respectively. Elevation in NSE activity was detected from the four field sites tested with either the αNa or the *p*-NPA substrates. With αNa, the activity in the Kisumu-susceptible strain (0.096±0.048) ranged from 0.04 to >0.12 µmol α-naphthol produced min^{-1} mg^{-1} protein. The same range was observed in most of the field samples. However, the distribution in the Douala and Mbalmayo samples was significantly different from that of the Kisumu strain and the Pitoa sample ($P<0.03$) due to a greater proportion of individuals with a high level of activity. Approximately 50% of individuals from the Douala and Mbalmayo samples displayed more than 0.1 µmol of α-naphthol produced $min^{-1}mg^{-1}$ protein, versus 17% and 30% in the Pitoa sample and the Kisumu-susceptible strain, respectively. The Simatou sample showed a low level activity (0.063±0.014, $P<0.001$), ranging from 0.04 to 0.1 µmol of α-naphthol min^{-1} mg^{-1} protein.

Table 1. Non-specific esterase (NSE), mixed function oxidase (MFO), and glutathione S-transferase (GST) activity (mean ± SD) in *Anopheles gambiae* s.l. tested populations.

Population	No. tested	*α*-Na	*p*-NPA	P_{450} Cyt.	GST
Kisumu	60	0.096 ± 0.048	0.041 ± 0.074	0.027 ± 0.011	0.055 ± 0.067
Douala	30	**0.102** ± 0.028	**0.051** ± 0.023	0.016 ± 0.010	**0.156** ± 0.030
Mbalmayo	31	**0.109** ± 0.032	0.007 ± 0.011	0.026 ± 0.014	0.030 ± 0.044
Pitoa	30	0.088 ± 0.061	**0.169** ± 0.092	**0.055** ± 0.026	0.044 ± 0.074
Simatou	30	0.063 ± 0.014	**0.057** ± 0.021	0.030 ± 0.010	0.039 ± 0.040

α-Na : NSE activity with substrate alpha-naphthyl acetate (µmol *α*-naphthol produced/min/mg protein).
p-NPA : NSE level with substrate para-nitrophenyl acetate (µmol *p*-nitrophenol produced/min/mg protein).
P_{450} Cyt. : MFO level (nmol EU/ mg protein).
GST : GST level (µmol GSH conjugated/min/mg protein).
Number in bold indicated samples where enzyme level was significantly higher compared with the Kisumu reference susceptible *An. gambiae* s.s. strain reared in our laboratory ($P<0.05$).

With *p*-NPA, the level of NSE activity in the Kisumu-susceptible reference strain (0.041±0.074) was lower than with α-NA (P<0.001), ranging from 0.0 to 0.05 μmol p-nitrophenol produced $min^{-1}mg^{-1}$ protein. The pattern of activity in all the field samples was significantly different from that of the Kisumu reference strain. The range in the Mbalmayo sample was similar to that of the Kisumu strain, but the mean value in the Mbalmayo sample (0.007±0.011) was very low (P<0.001), with around 70% of the individuals displaying activity less than 0.01 μmol of p-nitrophenol produced $min^{-1}mg^{-1}$ protein. Samples from Pitoa and Simatou showed high levels of activity with *p*-NPA compared with the Kisumu strain (P<0.001), even though they were not significantly increased with *α*-NA. The highest activity was observed in individuals from Pitoa (0.169±0.092), with levels ranging from 0.06 to 0.4 μmol of *p*-nitrophenol produced $min^{-1}mg^{-1}$ protein. In the Douala sample, elevated NSEs levels were seen with *p*-NPA (P<0.001) as well as with α-NA.

Mixed function oxidases

The pattern of MFO levels in laboratory and field samples is shown in Figure 4. The MFO level in the Kisumu-susceptible strain (0.027±0.011) ranged from 0.01 to 0.07 nmol EU mg^{-1} protein. The same range was seen in most of the field samples except those from Pitoa. The average in the Douala and Mbalmayo samples (0.016±0.010, 0.026±0.014) was lower than that of the Kisumu-susceptible reference strain (P<0.03), while the level in the Simatou sample (0.030±0.010) was not significantly different from that of the Kisumu strain (P=0.5). A significantly high level of MFOs was observed only in the Pitoa sample (0.055±0.026, P<0.001), where 28% individuals displayed values between 0.07 and 0.1 nmol EU mg^{-1} protein, much higher than that of the Kisumu strain.

Glutathione S-transferases

The distributions of the GST activity in tested samples are shown in Figure 5. The overall amount of conjugated GSH ranged from 0.0 to 0.24 μmol mg^{-1} protein. The value for the Kisumu-susceptible strain (0.055±0.067) was not significantly different from that observed in the Mbalmayo, Simatou, and Pitoa samples (P>0.05). However, GSH was expressed as a 2.8 fold increase in the Douala sample (P<0.001), ranging from 0.10 to 0.24 μmol mg^{-1} protein.

DISCUSSION

Insect resistance to insecticides is described by a number of patterns that are linked to biological responses through exposure to chemicals, and subsequently to genetic factors including biochemical or molecular mechanisms. A large-scale survey carried out between 1997 and 2001 indicated that numerous populations of *An. gambiae* s.l. from Cameroon were still susceptible to pyrethroid insecticides (Etang et al. 2003). However, DDT resistance was detected in areas where this insecticide was used during the 1950s for indoor residual spraying (Livadas et al. 1958, Cavalié and Mouchet 1962), whereas pyrethroid resistance occurred mostly in cotton areas. Mosquito samples used in this study were from specific sites where DDT or pyrethroid resistance was found, and they were previously used as controls during the process of insecticide susceptibility tests. These mosquitoes were not identified to species or molecular forms prior to biochemical analysis. However, specimens from the same collection which survived to insecticide exposure were identified and depicted for the *kdr* Leu-Phe allele.

The composition of these resistant individuals was 100% *An. arabiensis* for the Simatou sample, 59%/41% *An. arabiensis*/*An. gambiae* s.s. S form for the Pitoa sample, 100% *An. gambiae* s.s. predominantly M form (95%) for the Mbalmayo sample, and 100% *An. gambiae* s.s. M form for the Douala sample. These results are in agreement with the distribution of species and molecular forms within *An. gambiae* s.l. populations from Cameroon (Wondji et al. 2005). More interestingly, none of them displayed the *kdr* Leu-Phe mutation commonly involved in DDT and pyrethroid cross resistance (Etang et al. 2003), suggesting involvement of other resistance mechanisms.

Biochemical analyses were therefore used to describe the spectrum of metabolic activity in these resistant populations. Different profiles were seen for the three enzyme systems assayed compared to the Kisumu-susceptible strain of *An. gambiae*. High level NSEs appeared to be widespread while elevated MFOs and GSTs levels were only found in the Pitoa pyrethroid-resistant and the Douala DDT-resistant populations. In mosquitoes, esterases involved in resistance to insecticides can be active with both *α*- and *β*-Naphthyl acetate substrates. In this study, we used *α*-Naphthyl acetate subtrate, and we also used paranitrophenyl acetate because some esterases such as malathion carboxylesterases act preferentially on this substrate. Accordingly, NSE acted on *α*-Na in the Mbalmayo DDT-resistant sample, while acting on *p*-nitrophenyl acetate in the Simatou DDT-resistant and permethrin-tolerant sample as well as in the Pitoa pyrethroid-resistant sample. The Simatou and Pitoa mosquito populations were totally or partially comprised of *An. arabiensis* (Etang et al. 2003, Wondji et al. 2005), and this species was reported to display high levels of malathion carboxylesterase enzymes in Soudan (Hemingway 1985). An elevated level of MFOs was coupled with that of NSE in Pitoa where pyrethroids, organophosphate, and carbamate insecticides are widely used for cotton treatment. Elevation in GST activity was coupled with that of NSEs in the Douala town, where coils and mats containing pyrethroids are extensively used for protection against mosquito bites (Desfontaines et al. 1989), and subsequently to house spraying with DDT during the 1950s (Livadas et al. 1958). These results suggest that the increase in NSE, MFO, or GST levels may be related to extensive use of insecticides in the field. Current data are consistent with those reported in West Africa where the use of insecticides in agriculture and urban areas was found to play an important role in the resistance of *An. gambiae* s.l. to pyrethroids (Diabate et al. 2002). An increase in detoxification enzyme activity is therefore seen

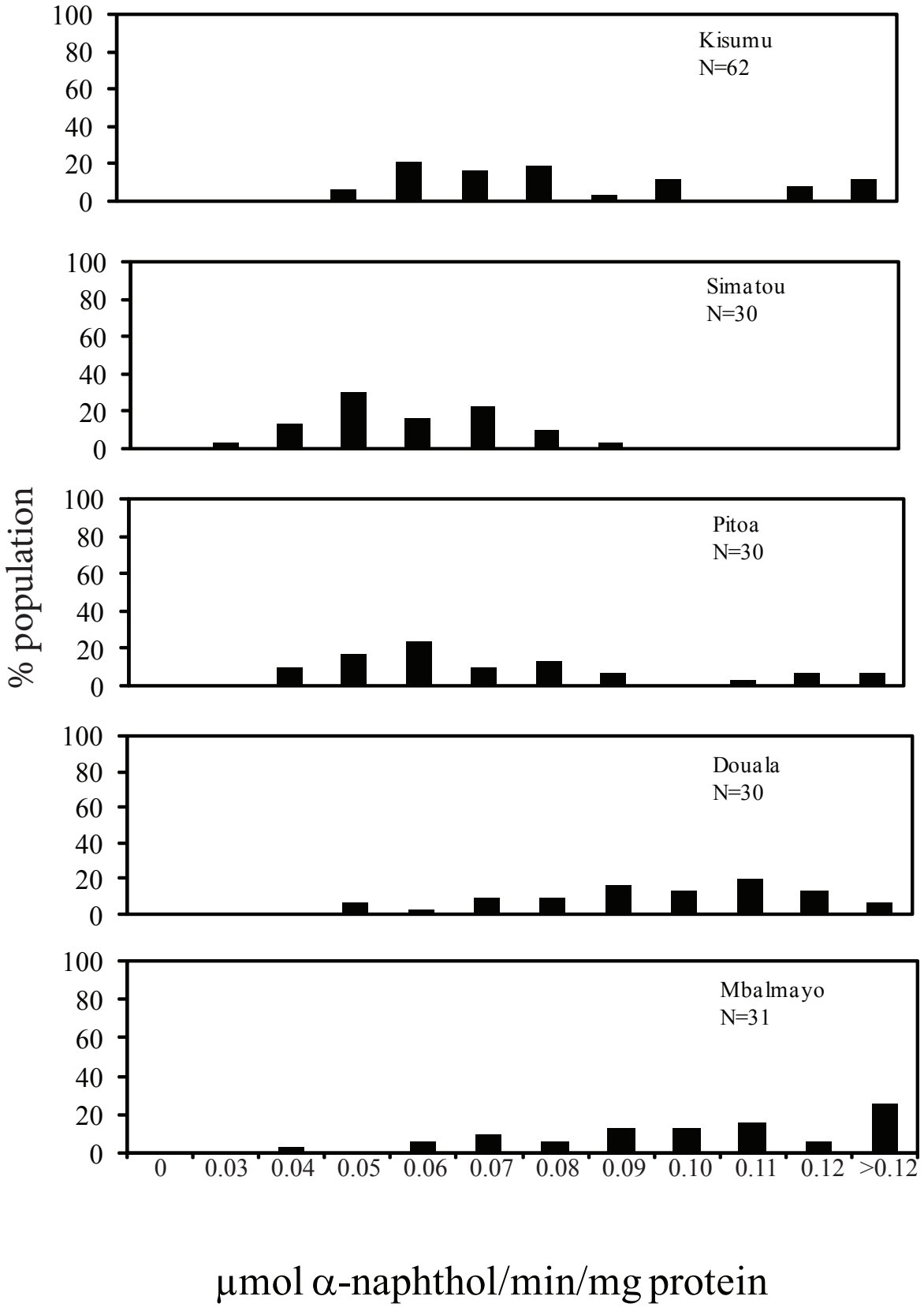

Figure 2. Non-specific esterase (NSE) profiles in the Kisumu-susceptible *Anopheles gambiae* s.s. strain and four wild populations with the substrate α-naphthyl acetate.

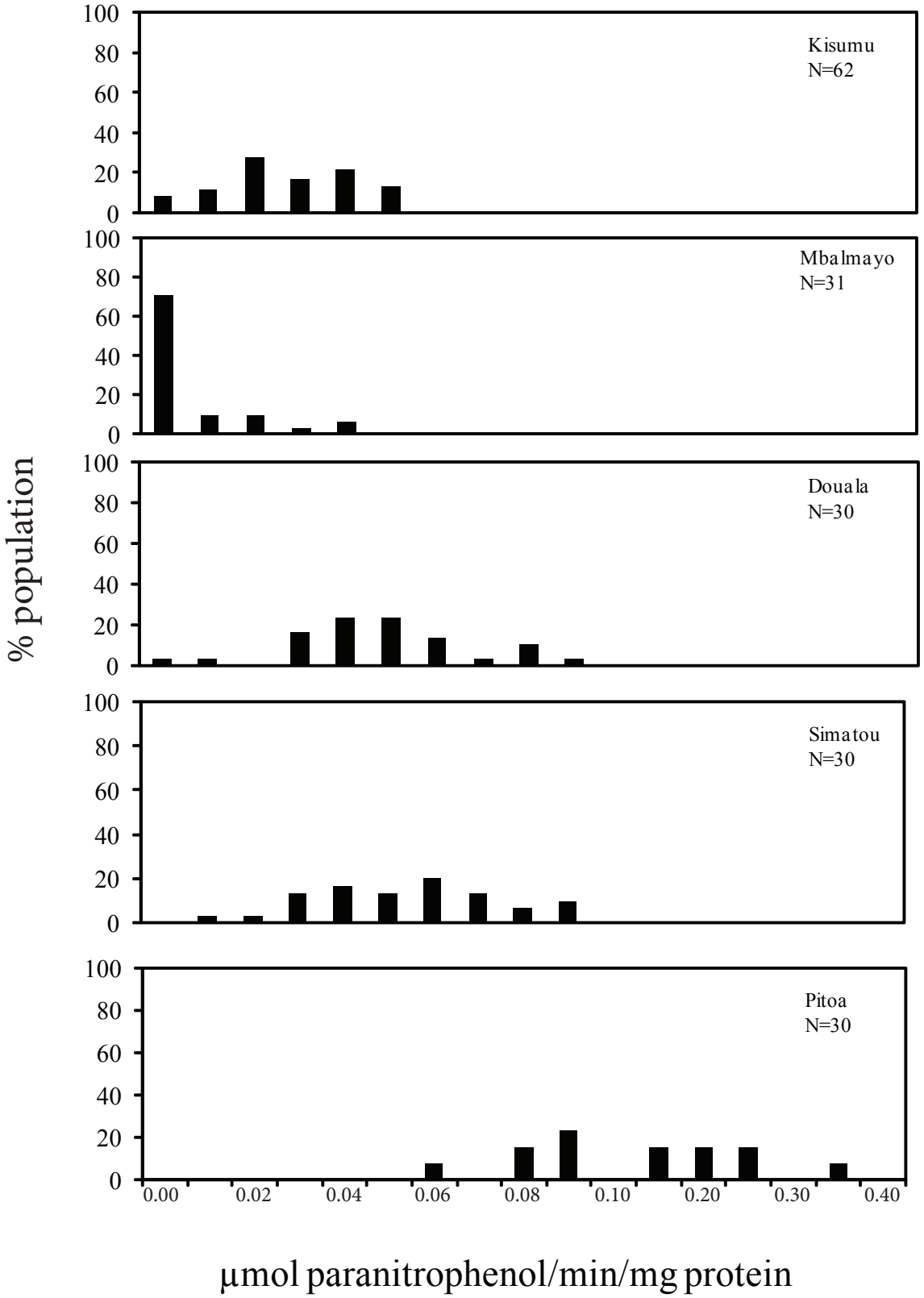

Figure 3. Non-specific esterase (NSE) profiles in the Kisumu-susceptible *Anopheles gambiae* s.s. strain and four wild populations with the substrate paranitrophenyl acetate.

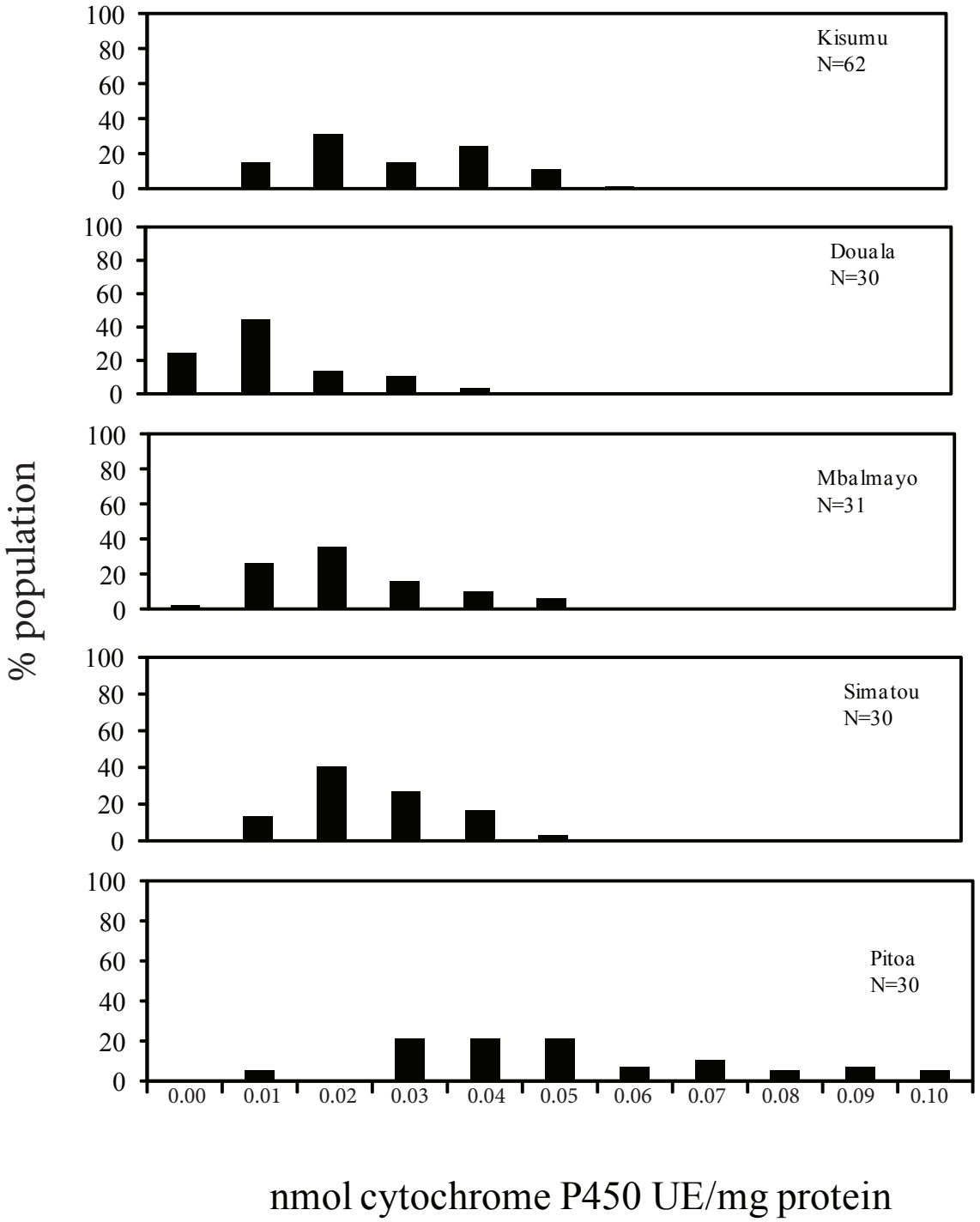

Figure 4. Mixed function oxidase (MFO) profiles in the Kisumu-susceptible *Anopheles gambiae* s.s. strain and four wild populations.

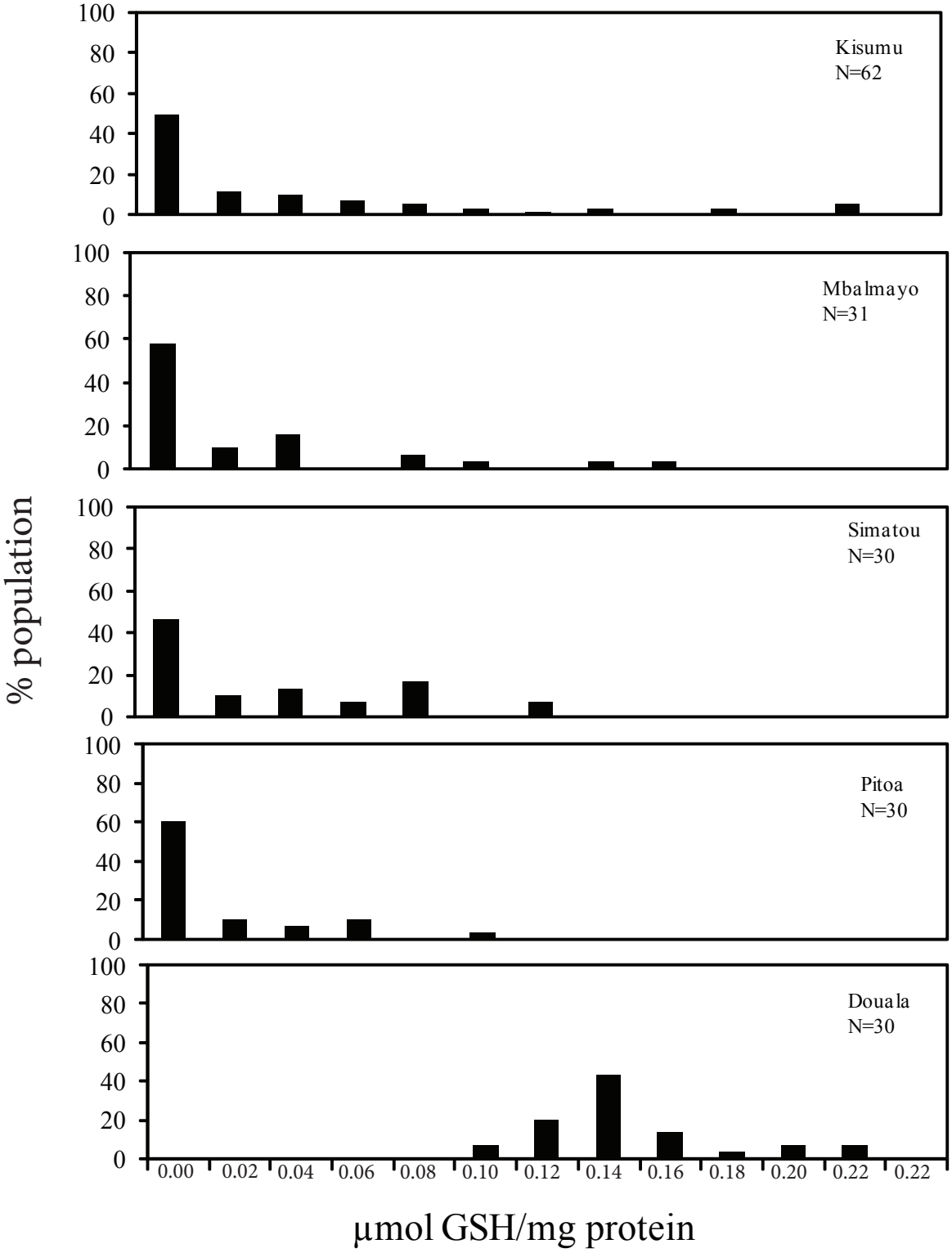

Figure 5. Glutathione S-transferase (GST) profiles in the Kisumu-susceptible *Anopheles gambiae* s.s. strain and four wild populations.

as a major DDT or pyrethroid resistance mechanism in *An. gambiae* s.l. populations from Cameroon.

Moreover, these findings suggest multiple or cross resistance in the tested populations. Other DDT or pyrethroid resistance mechanisms may also emerge in these areas, especially in Douala where the *kdr* Leu-Phe was recently observed at low frequency (Etang et al. 2006) soon after the current survey. However, the impact on specific insecticides remains to be explored by a correlation between enzyme activity and bioassay mortality, inhibition with enzyme inhibitors, and depiction of both *kdr* mutations.

With regard to the complexity of insecticide mode of action and genetic polymorphism in vector species, resistance mechanisms may differ from one vector species to another or within the same species from one population to another. In most of the tested samples we observed a broad spectrum of enzyme activities, showing that these samples were heterogeneous. The existence of two non-panmictic molecular M and S forms within *An. gambiae* s.s. species in southern Cameroon was previously reported (Wondji et al. 2002) and both forms as well as sympatric *An. arabiensis* in the northern area were resistant to insecticides (Etang et al. 2003). The segregation of the *kdr* Leu-Phe mutation in West Africa (Chandre et al. 1999b) and the *kdr* Leu-Ser mutation in East Africa (Ranson et al. 2000) is an example of genetic variation in resistance mechanisms. The distribution of the *kdr* Leu-Phe mutation in M and S molecular forms of *An. gambiae* s.s. from West Africa also provided evidence of reproductive isolation between the two forms (Chandre et al. 1999a, Diabate et al. 2003). This genetic barrier could lead to a differentiation of the biology and physiology of each species or molecular form, inducing different patterns of insecticide metabolism through enzyme systems. Subsequent studies are therefore needed to determine the involvement of each species and each molecular or chromosomal form of the *An. gambiae* complex in the process of metabolic-based resistance to insecticides.

Overall, the *kdr* Leu-Phe and Leu-Ser mutations have recently been reported in the western and coastal areas of Cameroon (Etang et al. 2006). Data from the current study provide baseline knowledge and emphasize the need for thorough investigations of metabolic resistance in *An. gambiae* s.l. populations from this country, taking into account the possibility of multiple resistance development.

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