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Monooxygenase Levels and Knockdown Resistance (*kdr*) Allele Frequencies in *Anopheles gambiae* and *Anopheles arabiensis* in Kenya

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ABSTRACT Pyrethroid-treated bed-nets and indoor spray are important components of malaria control strategies in Kenya. Information on resistance to pyrethroid insecticides in *Anopheles gambiae* and *An. arabiensis* populations is essential to the selection of appropriate insecticides and the management of insecticide resistance. Monooxygenase activity and knockdown resistance (*kdr*) allele frequency are biochemical and molecular indicators of mosquito resistance to pyrethroids. This study determined baseline information on monooxygenase activity and *kdr* allele frequency in anopheline mosquitoes in the western region, the Great Rift Valley–central region, and the coastal region of Kenya. In total, 1,990 field-collected individuals, representing 12 *An. gambiae* and 22 *An. arabiensis* populations were analyzed. We found significant among-population variation in monooxygenase activity in *An. gambiae* and *An. arabiensis* and substantial variability among individuals within populations. Nine of 12 *An. gambiae* populations exhibited significantly higher average monooxygenase activity than the susceptible Kisumu reference strain. The *kdr* alleles (L1014S) were detected in three *An. gambiae* populations, and one *An. arabiensis* population in western Kenya, but not in the Rift Valley–central region and the coastal Kenya region. All genotypes with the *kdr* alleles were heterozygous, and the conservative estimation of *kdr* allele frequency was below 1% in these four populations. Information on monooxygenase activity and *kdr* allele frequency reported in this study provided baseline data for monitoring insecticide resistance changes in Kenya during the era when large-scale insecticide-treated bed-net and indoor residual spray campaigns were being implemented.

KEY WORDS *Anopheles gambiae*, *Anopheles arabiensis*, monooxygenase, *kdr*, insecticide resistance

Insecticide-based vector control is a key tactic in the control and prevention of malaria, which results in 500 million clinical cases and 1 million deaths annually, with >90% of deaths occurring in sub-Saharan Africa (Greenwood and Mutabingwa 2002). Inexpensive insecticides such as DDT and dieldrin, used for indoor residual spray during the global malaria eradication campaign in the 1950s and 1960s, were initially very effective in many countries, including Kenya (Shanks et al. 2005). However, the effectiveness of DDT and dieldrin was not sustained, partially because of the emergence of resistance to the insecticides in the mosquito vectors (Akogbeto et al. 2006). Since the 1990s, pyrethroids have been promoted as insecticides of choice for bed-net impregnation and indoor spray (WHO 1993) due to their high efficacy, rapid rate of knockdown, strong mosquito excito-repellency, and low mammalian toxicity (WHO 1993, Hemingway et al. 2004, Lengeler 2004, Liu et al. 2006). As demonstrated in multisite trials throughout Africa, the large-scale use of insecticide-treated bed-nets (ITNs) reduces childhood malaria morbidity by up to 50% and overall mortality by 20–30% in Gambia, Ghana, Kenya, and Tanzania (Alonso et al. 1991; Binka et al. 1996; Nevill et al. 1996; Phillips-Howard et al. 2003; Killeen et al. 2006, 2007).

Consequently, WHO recommends the large-scale use of ITNs to control malaria transmission because ITNs offer a good cost-effective method based on active community involvement (Wiseman et al. 2003). Currently, pyrethroid-treated bed-nets and indoor spray are the central components in the Global Strategy for Malaria Control and the Roll Back Malaria program, which was launched in 1998 (WHO 1993, Yamey 2004), and more recently in the malaria control programs funded by the President's Malaria Initiative (PMI 2005) and the Global Fund to Fight against AIDS, Tuberculosis, and Malaria (GFATM 2007). With increased use of pyrethroid insecticides for malaria vector control in Africa, resistance to pyrethroids was reported in the major African malaria vectors, including *Anopheles gambiae* in Benin, Burkina Faso, Ivory Coast, Kenya and Mozambique (Elissa et al. 1993, Vulule et al. 1994, Curtis et al. 1998, Chandre et al. 2000, Casimiro et al. 2006a), and *Anopheles funestus* in Mozambique and South Africa (Hargreaves et al.

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2000, Brooke et al. 2001, Casimiro et al. 2006b). The distribution of mosquito resistance is uneven, with greater resistance reported in western and southern Africa than in eastern Africa.

Anopheline mosquitoes exhibit two major mechanisms of pyrethroid resistance through an increased level of metabolic detoxification of the insecticide and reduced sensitivity in the target sites of the insecticide. Metabolic detoxification is generally associated with increased monooxygenase activity, causing not only pyrethroid resistance but also cross-resistance to unrelated compounds (Bergé et al. 1998, Liu et al. 2006). The target site of pyrethroids is the voltage-gated sodium channel. A point mutation in the region II of the *para*-type sodium channel gene causes a change in affinity between the insecticide and its binding site on the sodium channel, and it induces a phenotype termed knockdown resistance (*kdr*) in a range of insects (Williamson et al. 1996; Dong 1997; Jamroz et al. 1998; Martinez-Torres et al. 1998, 1999; Ranson et al. 2000; Sina and Aultman 2001). Insensitivity at the sodium channel target sites also leads to cross-resistance between different classes of insecticides (Brengues et al. 2003). Permethrin knock-down resistance has arisen independently at least twice in Africa. In west Africa, *kdr* was caused by a leucine-phenylalanine substitution at position 1014 of the sodium channel gene (L1014 F), whereas a different mutation (leucine-serine substitution) at the same amino acid position (L1014S) was associated with *kdr* resistance in Kenya in both *An. gambiae* and *An. arabiensis* (Martinez-Torres et al. 1998, Ranson et al. 2000, Stump et al. 2004). However, the L1014 F mutation has been detected in *An. gambiae* and *An. arabiensis* in Tanzania and Uganda (Kulkarni et al. 2006, Verhaeghen et al. 2006). Interestingly, both L1014 F and L1014S mutations were detected in the *An. gambiae* mosquitoes from Libreville in Gabon, and the L1014S mutation exhibited a higher frequency than L1014 F (Pinto et al. 2006).

The objective of the current study is to determine broad-scale baseline information on pyrethroid resistance in major malaria vectors in Kenya. Malaria vectors could be exposed to selection by insecticides through previous insecticide use for agricultural or public health pest control. Thus, information on the current resistance level is valuable in predicting the rate of pyrethroid resistance increase in the era of large-scale use of pyrethroid insecticides for malaria vector control, and in designing appropriate strategies to manage the resistance. We used mosquito monooxygenase levels and *kdr* allele frequency as resistance surrogates. Mosquito resistance as detected by biochemical assays could be influenced by age, blood feeding, sex, body size, and other physiological statuses such as preexposure to insecticides and mating (Vulule et al. 1999; Hunt et al. 2005; Casimiro et al. 2006a, b; Matambo et al. 2007). Therefore, in this study, we first determined the effects of age and blood feeding on individual monooxygenase levels in a susceptible *An. gambiae* strain because this enzyme is expressed as equivalent units of cytochrome P450. With the established methods of biochemical measurement and polymerase chain reaction (PCR) detection, we determined the baseline information on monooxygenase levels and *kdr* allele frequencies of *An. gambiae* and *An. arabiensis* populations in three ecological and epidemiologically different zones in Kenya.

Materials and Methods

Study Areas and Specimen Collection. Between April and June 2005, mosquito larvae were collected from 34 sites in the three geographically and epidemiologically distinct regions, the western region (16 sites), the Great Rift Valley and central region (12 sites), and the coastal region (six sites) in Kenya (Fig. 1; Table 1). In western Kenya, particularly the basin region of Lake Victoria, malaria transmission is perennial, and transmission intensity measured by entomological inoculation rate (EIR) is typically in the order of several dozens to several hundreds infectious bites per person per year, and *An. gambiae* is the predominant malaria vector (Chen et al. 2004, Shanks et al. 2005). Malaria transmission in the Great Rift Valley is seasonal, *An. arabiensis* is the predominant malaria vector, and *An. gambiae* has not been reported in the region (Lehmann et al. 2003, Nyanjom et al. 2003, Temu and Yan 2005). In the central region, malaria transmission is sporadic, and *An. arabiensis* is also the predominant malaria vector (Chen et al. 2006a). Malaria transmission in coastal Kenya is perennial, but transmission intensity is generally much lower than in western Kenya, and *An. gambiae* is the predominant malaria vector species (Mbogo et al. 2003).

Larvae of *An. gambiae* s.l. were collected using the standard 350-ml dippers, kept in plastic bottles, and transported to an insectary closer to a collection site. The collection sites are categorized into five types: small pond (created by rain or spring water of several square meters and no full coverage of aquatic plants), big pond (standing water body of >50 m^2 without full coverage of aquatic plants), swamp (various sizes of water bodies with high density of aquatic plants), footprints (animal or human footprints with rain water), and swimming pool (drained concrete pool with shallow rainwater at the bottom). Larvae were reared to adults with TetraMin fish food for quantification of monooxygenase activities. The global coordinates of all collection sites were recorded using a handheld global positioning system unit. We did not collect adult female mosquitoes from the field because the measurement of monooxygenase activity may be strongly affected by mosquito age that cannot be determined reliably.

A susceptible laboratory colony of *An. gambiae* s.s. (the WHO-referenced Kisumu strain; Vulule et al. 1996, 1999) was used to establish baseline enzyme activities in susceptible mosquitoes and to determine whether the monooxygenase activity in adult mosquitoes varied by age and food types. One-day-old female adult mosquitoes were divided into two groups. Group

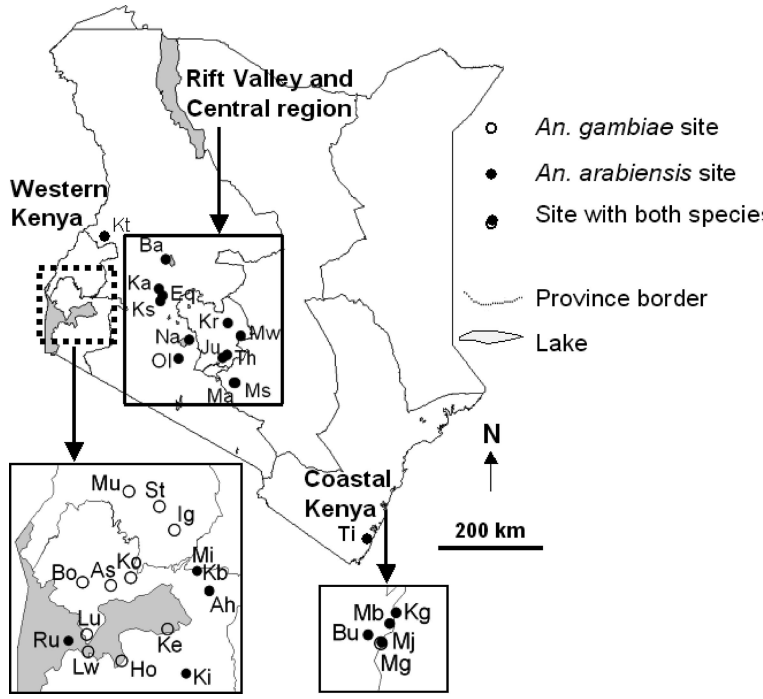

Fig. 1. Schematic map of collection sites. Site codes are defined in Table 1. Circles and solid circles indicate the sites where at least 40 *An. gambiae* and *An. arabiensis* larvae were collected, respectively.

1 was fed with 6% sucrose solution for 30 min and then maintained on water without sucrose (sugar-fed group). Group 2 was exposed to restrained rabbits for 30 min; only fully engorged mosquitoes were maintained on water without sucrose and used for monooxygenase activity measurement (blood-fed group). Twenty five to 30 female mosquitoes of the Kisumu strain were tested for monooxygenase activity every 1–2 d for a total of 22 d.

Monooxygenase Quantification. Monooxygenase activity was measured in individual female mosquitoes, by using the method described by Brengues et al. (2003), with a slight modification. Briefly, a substrate solution of 20 mM 7-ethoxycoumarine (7-EC) was prepared in ethanol, and 20 μl of the 7-EC solution was added to 1 ml of 50 mM phosphate buffer, pH 7.2, containing 1 mM EDTA, 0.1 mM dithiothreitol, 2 mM 1-chloro-2, 4-dinitrobenzene, and 0.1 mM reduced glutathione. Individual females were homogenized in a 0.5-ml tube with 150 μl of the 7-EC buffer solution. After homogenizing the mosquitoes, samples were spun and the supernatant was used for monooxygenase assay. The pellet was preserved for DNA isolation. After incubation at 30°C for 4 h, the conversion reaction of 7-EC to 7-hydroxycoumarine was stopped by adding 210 μl of 0.1 mM glycine buffer, pH 10.4. The solution was transferred into a cuvette without mosquito tissue, and optical density (OD) at 450 nm was measured using a SMART Spectro spectrophotometer (LaMotte Co., Chestertown, MD). The OD values were converted into concentration (picograms per microliter) by using a standard regression based on a serial dilution of 7-hydroxycoumarine (Kasei Kogyo Co., Tokyo, Japan) and its relevant OD values. Forty to 90 adult females from field collections, at postemergence ages of 7–10 d, were tested for monooxygenase activity. We chose 7–10-d-old adult mosquitoes for monooxygenase activity determination because the enzyme level was not stable in younger mosquitoes (see below). In total, 1,990 individuals were analyzed and reported in this study.

DNA Extraction, Species Identification, and *kdr* Detection. Genomic DNA was extracted individually from the preserved mosquito tissue after monooxygenase measurement, following the previously described method (Chen et al. 2004, 2006b). Field collected samples within *An. gambiae* s.l. were further identified to species using the rDNA-polymerase chain reaction (PCR) method described by Scott et al. (1993). If a sample could not be identified after three PCR amplifications, it was marked as unknown. The *kdr* genotypes were determined for all specimens, using the allele-specific PCR assay described by Ranson et al. (2000). In total, 1990 individuals were tested for *kdr* alleles.

ND5 Gene Sequencing. Because the above-tested samples were collected at larval stage from one or several nearby aquatic sites at each location, there was a possibility that the resistant individuals from the same or several closely located breeding sites may have been maternally related (Chen et al. 2006b). To determine the maternal relatedness of individuals with the *kdr* allele, a 665-bp polymorphic fragment of the subunit five of the mitochondrial NADH dehy-

Table 1. Collection sites in Kenya, larval habitat types, and sample size used in this study

Region	Site (abbreviation)	Types of larval sampling site	No. *An. gambiae*	No. *An. arabiensis*
Western	Rusinga (Ru)	Small ponds	—[a]	40
	Bondo (Bo)	Swamp	50	—
	Luanda (Lu)	Swamp	49	—
	Lwanda (Lw)	Swamp	47	—
	Asembo (As)	Footprints	50	—
	Homa Bay (Ho)	Small ponds and swamp	50	—
	Mumias (Mu)	Small ponds	67	—
	Kombewa (Ko)	Small ponds	66	—
	Stendmwako (St)	Small ponds	66	—
	Kendo (Ke)	Small ponds	90	—
	Iguhu (Ig)	Small ponds	56	—
	Kisii (Ki)	Small ponds	—	50
	Miwani (Mi)	Small ponds	—	70
	Ahero (Ah)	Footprints	—	54
	Kibigori (Kb)	Small ponds	—	70
	Kitale (Kt)	Small ponds	—	64
Rift Valley and Central	Katheluny (Ka)	Swamp	—	44
	Kibshana (Ks)	Big ponds	—	52
	Equator (Eq)	Big ponds	—	56
	Baringo (Ba)	Small ponds	—	56
	Olasiti (Ol)	Small ponds	—	60
	Naivasha (Na)	Small ponds	—	60
	Juja (Ju)	Small ponds	—	66
	Thika (Th)	Small ponds and swamp	—	58
	Karatina (Kr)	Small ponds	—	58
	Machakos Stream (Ma)	Small ponds	—	66
	Machakos Swamp (Ms)	Swamp	—	80
	Mwea (Mw)	Footprints	—	70
Coastal	Tiwi (Ti)	Swamp	58	—
	Burangi (Bu)	Footprints	—	56
	Malindi Green (Mg)	Swimming pool	60	—
	Malindi Baob (Mb)	Swimming pool	—	56
	Majejena (Mj)	Swamp	—	50
	Kangombani (Kg)	Small ponds	—	45

[a] —, mosquito samples at a particular site were either not present or had insufficient numbers (<40) for monooxygenase and *kdr* analyses.

drogenase gene (ND5) was amplified using primer 19CL and DMP3A (Besansky et al. 1997), and it was sequenced from both ends by using the ABI BigDye Sequencing kit (Applied Biosystems, Foster City, CA). In total, 39 *An. gambiae* individuals with the L1014S mutation in the sodium channel gene collected in three sites (Bondo, Mumias, and Stendmwako) were sequenced.

Data Analyses. The collection sites were mapped with ArcView 3.2 GIS software. The OD values of monooxygenase level were converted into concentration in picogram per minute per larva, based on the standard curve. One-way analysis of variance (ANOVA) with age as the factor was conducted for the laboratory Kisumu *An. gambiae* strain to determine whether monooxygenase activity varied with mosquito age. Monooxygenase activity data were square root transformed. The *t*-test was used for group comparison when appropriate. One-way ANOVA also was used to examine whether monooxygenase activity varied among the sampling sites for *An. gambiae* and *An. arabiensis.* The sampling site was treated as a random factor. A chi-square test was applied to test whether *kdr* frequencies varied significantly among the sampling sites. The ND5 gene sequence data were aligned and haplotypes identified using the BioEdit software (Hall 1999).

Results

Monooxygenase Activity in the Susceptible *An. gambiae* Reference Strain. Monooxygenase activity varied significantly with mosquito age in both the sugar-fed ($F = 5.62$; df = 12, 289; $P < 0.01$) and blood-fed ($F = 8.44$; df = 12, 293; $P < 0.01$) groups of the susceptible Kisumu strain. The enzyme activity in certain mosquitoes increased dramatically 1–4 d after sugar or blood feeding, and then it gradually decreased and remained temporally stable (Fig. 2). We termed the period of dramatic increase in monooxygenase activity of certain individuals the "stimulation phase" and the temporally less variable period the "stable phase." The sugar-fed group took 7 d to reach the stable phase, whereas the blood-fed group took ≈10 d (Fig. 2). The enzyme levels in the stimulation phases were significantly higher than those in the stable phases in both sugar-fed group ($t = 4.04$, df = 116, $P < 0.01$) and blood-fed group ($t = 6.43$, df = 105, $P < 0.01$). Moreover, within each group, there was more variability in monooxygenase activity during the stimulation phase than the stable phase (Fig. 2). The sugar-fed and blood-fed groups did not differ significantly in the enzyme level in the stimulation phase ($t = 0.77$, df = 206, $P = 0.44$) and stable phase ($t = 1.92$, df = 336, $P = 0.06$), suggesting that the blood feeding did not affect monooxygenase activities.

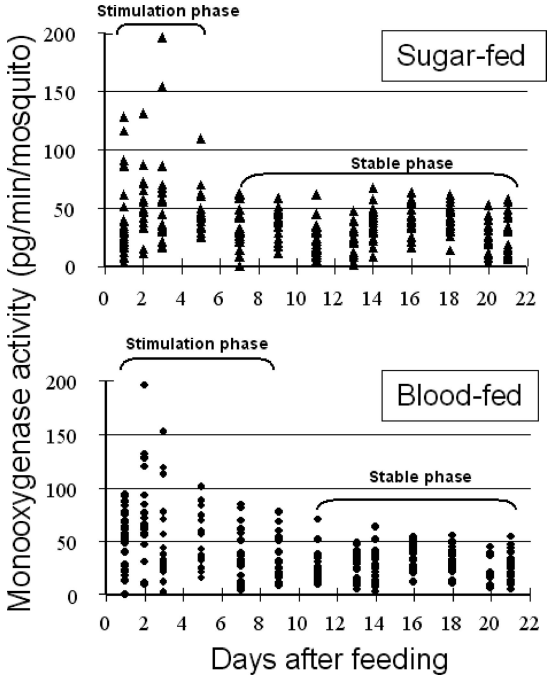

Fig. 2. Dynamics of monooxygenase activity in sugar-fed and blood-fed *An. gambiae* females.

Monooxygenase Levels in Field-Collected *An. gambiae* and *An. arabiensis*. Using sugar-fed adults at 7–10 d after emergence (in the stable phase), we detected considerable within-population variations in monooxygenase activity for both wild *An. gambiae* and *An. arabiensis* (Fig. 3). Such a large within-population variation contrasts the susceptible Kisumu *An. gambiae* reference strain. Both species exhibited significant among-site variation in monooxygenase activity (*An. gambiae:* $F = 80.85$; df = 11, 697; $P < 0.001$ and *An. arabiensis:* $F = 58.51$; df = 21, 1,259; $P < 0.001$). In particular, *An. gambiae* populations from Iguhu (west Kenya) and Tiwi (coastal Kenya) showed the highest values of monooxygenase activity (>90 pg/min per adult), whereas those from Mumias and Stendmwako (west Kenya) exhibited the lowest values (<30 pg/min per adult) (Fig. 3). *An. arabiensis* populations from Machakos (Rift Valley) had the highest monooxygenase levels (>90 pg/min per adult), whereas those from Ahero and Kibigori (west Kenya) showed the lowest value (<30 pg/min per adult) (Fig. 3). Compared with the susceptible Kisumu reference strain of the same age (sugar-fed, 7 and 9 d old), the field-collected *An. gambiae* at most sites exhibited substantially higher monooxygenase activity ($F = 80.10$; df = 12, 739; $P < 0.001$) except at Mumias (Mu) and Malindi Green (Mg), where the *An. gambiae* enzyme activity showed no significant difference from that of the Kisumu strain, and at Stendmwako (St), where the *An. gambiae* enzyme activity was signifi-

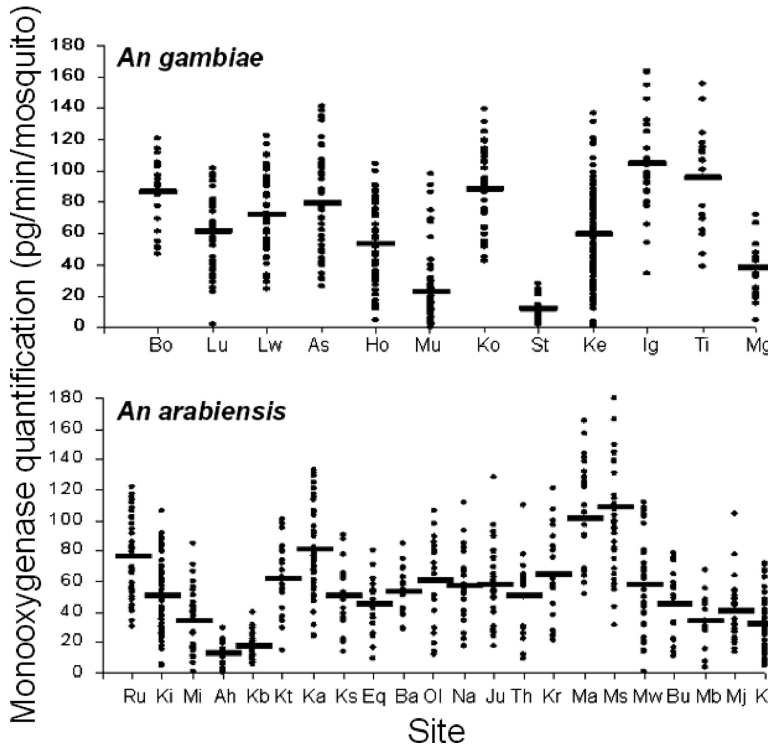

Fig. 3. Monooxygenase levels in *An. gambiae* and *An. arabiensis* populations. Site codes are defined in Table 1. Bars indicate population means of enzyme activities.

Table 2. *kdr* allele frequency in *An. gambiae* and *An. arabiensis* in western Kenya

Species	Site	*n*	No. heterozygous *kdr* genotypes	Observed *kdr* allele frequency (%)[a]	Conservative estimate of *kdr* allele frequency (%)[b]
An. gambiae	Bondo	50	15	15.0	1.0
	Mumias	67	17	12.7	0.7
	Stendmwako	66	7	5.3	0.8
An. arabiensis	Ahero	54	1	0.9	0.9

[a] Observed *kdr* allele frequency was calculated as the detected number of *kdr* allele divided by the total number of alleles in one population.
[b] Conservative estimate of *kdr* allele frequency was calculated based on the assumption of all individuals with heterozygous *kdr* genotype in a site were from one mother.

cantly lower than that of the Kisumu strain. Overall, monooxygenase activity in *An. arabiensis* from western Kenya was significantly lower than that from the Rift Valley–central region and coastal Kenya ($F = 129.07$; df = 2, 1,278; $P < 0.001$).

***kdr* Allele Frequencies in *An. gambiae* and *An. arabiensis*.** The L1014S-*kdr* allele was detected in three *An. gambiae* populations and one *An. arabiensis* population from western Kenya (Table 2). All the *kdr* alleles were presented in heterozygous states. The L1014S-*kdr* alleles were found in 39 of 709 *An. gambiae* mosquitoes. Of 1,281 *An. arabiensis* individuals tested, only one resistant allele in heterozygous state was detected in the Ahero population in western Kenya. Thus, the observed *kdr* allele frequencies in *An. arabiensis* were significantly lower than in *An. gambiae* in western Kenya ($\chi^2 = 10.22$, df = 1, $P < 0.01$). L1014 F-*kdr* allele was not detected in any population.

Because the analysis of *kdr* allele frequency was based on larval samples collected from larval habitats, the samples may have been be maternally related. We sequenced the 665-bp polymorphic region of ND5 for all 39 *An. gambiae* specimens with the L1014S-*kdr* allele, and we detected six polymorphic sites in two haplotypes (Table 3). Individuals with the *kdr* allele from Mumias and Stendwako had the identical haplotype. Similarly, all individuals with the heterozygous *kdr* genotype from Bondo showed a single haplotype. This result suggests that the *An. gambiae* mosquitoes of *kdr*-genotype from one larval breeding site were likely produced by the same female. Therefore, the conservative estimate of *kdr* allele frequency was <1% in all sites for both *An. gambiae* and *An. arabiensis* (Table 2).

Discussion

This study examined monooxygenase activity and *kdr* frequency distribution in the natural *An. gambiae* and *An. arabiensis* populations in western, Rift Valley–central, and coastal regions of Kenya because elevated monooxygenase activity and increased *kdr* frequency in a population are associated with increased resistance to pyrethroids (Hemingway et al. 2004, Liu et al. 2006). We found significant variation in monooxygenase activity among the sampling sites in both species, and we detected significant differences in the enzyme activity of *An. arabiensis* across the three regions. In comparison to the susceptible Kisumu *An. gambiae* strain, nine of 12 *An. gambiae* field-collected populations exhibited significantly higher monooxygenase activity. The *kdr* alleles were detected in three of 10 *An. gambiae* populations and one of six *An. arabiensis* populations in western Kenya, but they were not observed in the Rift Valley and coastal Kenya.

In the current study, we collected anopheline larvae from natural habitats and reared them to adults for monooxygenase activity and *kdr* frequency determination. The advantage of this approach was that all mosquito samples used in the biochemical test were at the same age; thus, age was not a confounding factor for comparisons of monooxygenase activity among localities or for examination of enzyme activity variability among individuals within a population. This is important because in the time course experiments with the susceptible laboratory *An. gambiae* colony, monooxygenase activity is age-dependent and less stable among young (<8 d after emergence) mosquitoes than older (>9 d after emergence) mosquitoes. The higher monooxygenase activity observed in the young mosquito group may be related to aging and mosquito feeding. Female mosquitoes used in our study were exposed to sucrose or rabbit bloodmeal. The release of reactive oxygen species during the digestion of bloodmeal (heme) can be toxic to mosquitoes without detoxification enzymes (Strode et al. 2006). Similarly, bacteria may be ingested during sucrose feeding; thus, mosquitoes may be exposed to bacterial toxin. Therefore, detoxification enzymes such as monooxygenase would be elevated after feeding. Strode et al. (2006) found that ≈25% of the detoxification genes are expressed at significantly different levels in the larva, pupal and adult stages. Our time course results on monooxygenase activity are consistent with the previous pyrethroid bioassays on *An. gambiae* and *An. stephensi* that the mean median knockdown times (KT_{50}) declined with mosquito age (Vulule et al. 1994; Hodjati and Curtis 1996). Interestingly, sugar feeding

Table 3. Polymorphic sites of *An. gambiae* mitochondrial DNA ND5 haplotypes observed in western Kenya

Sampling site	Nucleotide position[a]					
	6829	7027	7099	7108	7240	7360
Mumias and Stendwako	C	T	C	G	A	C
Bondo	A	C	T	A	G	T

[a] Nucleotide position is denoted based on the *An. gambiae* mitochondrial genome sequence, GenBank accession no. NC_002084.

or blood feeding did not exhibit any significant effect on monooxygenase activity. Thus, to measure monooxygenase activity in natural mosquito populations, either blood-fed or sugar-fed mosquitoes can be used as long as their ages reach the stable phase (Fig. 2). The disadvantage of the approach is that some samples from the same habitat may be genetically related (Chen et al. 2006b, 2008), leading to over- or underestimation of *kdr* allele frequency. For example, in the current study, the observed *kdr* allele frequencies were between 5 and 15% at three *An. gambiae* populations in western Kenya (Table 2). Because some *kdr*-allele individuals from an aquatic breeding site were likely from the same female, the conservative estimation of *kdr* frequency should be <1%. Several studies in western Africa *An. gambiae* populations found extremely high *kdr* allele frequency based on larval samples (e.g., 20–96% in Ivory Coast [Martinez-Torres et al. 1998] and 89–96% in Burkina Faso [Diabate et al. 2002]). We recommend using adult samples or using larvae from many diverse aquatic habitats for insecticide resistance surveillance to minimize sampling bias.

In the current study, the low *kdr* frequencies in multiple sites suggest that the selection pressure resulting from current and historic uses of insecticide has not led to a high *kdr* allele frequency in *An. gambiae* and *An. arabiensis* in Kenya. In the 1990s and early 2000s, ITN coverage in Kenya was generally low (Guyatt et al. 2004), and large-scale ITN coverage and indoor residual spray were primarily concentrated in bed-net trial areas or well-organized vector control areas (Nevill et al. 1996, Bogh et al. 1998, Hawley et al. 2003, Lindblade et al. 2006). Our survey, conducted during mosquito sampling, found that occasional agricultural insecticide use was primarily in the rice, coffee/tea plantation and sugar cane growing areas, consistent with other reports on the agricultural use of insecticides in Kenya (Kamau and Vulule 2006). Stump et al. (2004) reported that the frequency of the *kdr* allele in *An. gambiae* increased from 3–4% to 4–8% after 7–8 yr of using pyrethroid-treated bed-nets in an ITN trial area in Asembo Bay, west Kenya. Therefore, intensive use of ITNs selected for increase of *kdr* allele frequency. Information on monooxygenase activity and *kdr* allele frequency reported in the current study provided baseline data for monitoring insecticide resistance changes in Kenya in the era of large-scale ITN coverage being implemented by the Global Fund to Fight against AIDS, Tuberculosis, and Malaria (GFATM 2007).

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