Medical and Veterinary Entomology

Medical and Veterinary Entomology (2013) **27**, 276–283 doi: 10.1111/j.1365-2915.2012.01055.x

Insecticide resistance monitoring of field-collected *Anopheles gambiae s.l.* populations from Jinja, eastern Uganda, identifies high levels of pyrethroid resistance

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Abstract. Insecticide resistance in the malaria vector *Anopheles gambiae s.l.* (Diptera: Culicidae) threatens insecticide-based control efforts, necessitating regular monitoring. We assessed resistance in field-collected *An. gambiae s.l.* from Jinja, Uganda using World Health Organization (WHO) biosassays. Only *An. gambiae s.s.* and *An. arabiensis* (≈70%) were present. Female *An. gambiae* exhibited extremely high pyrethroid resistance (permethrin $LT_{50} > 2$ h; deltamethrin $LT_{50} > 5$ h). Female *An. arabiensis* were resistant to permethrin and exhibited reduced susceptibility to deltamethrin. However, while *An. gambiae* were DDT resistant, *An. arabiensis* were fully susceptible. Both species were fully susceptible to bendiocarb and fenitrothion. *Kdr 1014S* has increased rapidly in the Jinja population of *An. gambiae s.s.* and now approaches fixation (≈95%), consistent with insecticide-mediated selection, but is currently at a low frequency in *An. arabiensis* (0.07%). *Kdr 1014F* was also at a low frequency in *An. gambiae*. These frequencies preclude adequately-powered tests for an association with phenotypic resistance. PBO synergist bioassays resulted in near complete recovery of pyrethroid susceptibility suggesting involvement of CYP450s in resistance. A small number (0.22%) of *An. gambiae s.s.* × *An. arabiensis* hybrids were found, suggesting the possibility of introgression of resistance alleles between species. The high levels of pyrethroid resistance encountered in Jinja threaten to reduce the efficacy of vector control programmes which rely on pyrethroid-impregnated bednets or indoor spraying of pyrethroids.

Key words. *Anopheles arabiensis*, *kdr*, hybridization, introgression, malaria, metabolic resistance, permethrin.

Introduction

In this era of malaria control and elimination, the World Health Organization (WHO) is advocating a rapid scale-up of vector control interventions with major roles for insecticide-treated nets (ITNs) and indoor residual spraying (IRS) (WHO, 2011). However, only four classes of mosquito adulticides (organochlorines, pyrethroids, carbamates and organophosphates) are available for vector control (Nauen, 2007; Ranson *et al.*, 2011) and no new public-health insecticide has been licensed in the past two decades (Nauen, 2007; Kelly-Hope *et al.*, 2008). The four classes share just two target sites (pyrethroids and DDT target the voltage-gated sodium channel and carbamates and organophosphates target acetylcholinesterase) and with this severe limitation the selection pressure induced by control strategies is likely to lead to the development of resistance.

While the rotational use of insecticides from different classes in order to mitigate against resistance is feasible for IRS application, ITNs are reliant solely on pyrethroids

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owing to their low mammalian toxicity. While conclusive proof is presently lacking, there is some empirical evidence that insecticide resistance may reduce the efficacy of malaria vector control efforts (N'Guessan *et al.*, 2007; Sharp *et al.*, 2007; Ranson *et al.*, 2011; Asidi *et al.*, 2012). Resistance to pyrethroids in the most important African malaria vector *An. gambiae sensu stricto* Giles (della Torre *et al.*, 2002) has been widely reported in Sub-Saharan Africa (Ranson *et al.*, 2011) and recently in Uganda (Verhaeghen *et al.*, 2006, 2010; Rubaihayo *et al.*, 2008; Ramphul *et al.*, 2009). If insecticide resistance does impact upon control then this is a major concern for malaria control efforts in Uganda (Yeka *et al.*, 2012) and regular resistance monitoring will be pivotal in counteracting this threat.

Resistance to pyrethroid insecticides is associated predominantly with target site insensitivity and increased detoxification (Berge *et al.*, 1998; Ranson *et al.*, 2011). Two single base substitutions in the voltage-gated sodium channel commonly referred to as knockdown resistance (*kdr*) mutations that confer cross-resistance to DDT and pyrethroids have been described in *An. gambiae sensu lato* populations (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000). A leucine–serine substitution at position 1014 (*1014S*) identified originally in Kenyan *An. gambiae s.s.* (Ranson *et al.*, 2000) and referred to as *kdr-east* and a leucine–phenylalanine substitution at the same amino acid position (*1014F*) identified originally in West Africa (Martinez-Torres *et al.*, 1998) and referred to as *kdr-west* have a strong association with resistance (Donnelly *et al.*, 2009). The *1014S* variant is now found in *An. gambiae s.s.* throughout much of East/Central Africa (Etang *et al.*, 2006; Pinto *et al.*, 2006; Ridl *et al.*, 2008) and there has been a rapid rise in frequency of *1014S* in Kenyan *An. gambiae s.s.* coinciding with the scaling up of ITN distribution (Mathias *et al.*, 2011). While the *1014F* allele is at high frequency in West Africa it has only rarely been reported in East African populations including at a very low frequency in Ugandan *An. gambiae s.s*. (Verhaeghen *et al.*, 2006).

The *An. gambiae s.l.* species complex is regarded as the primary target for ITN and IRS vector control strategies in Uganda (Ugandan MOH, 2010) and previous studies have detected pyrethroid and DDT resistance in these vectors in Uganda (Ramphul *et al.*, 2009; Verhaeghen *et al.*, 2010). In recent work in Apac, northern Uganda, resistance of the local *An. gambiae* population to pyrethroid insecticides and DDT prompted a switch to a carbamate insecticide to which no resistance had been detected. While IRS using DDT in the first round of spraying and the class II pyrethroid α-cypermethrin in the second round gave rise to a modest (but significant) decrease in malaria morbidity in the <5 years age group [odds ratios (ORS) of 0.76 and 0.83, respectively], when IRS switched to bendiocarb to which the mosquito population was fully susceptible, the decrease in malaria morbidity was much greater (OR = 0.16–0.34 over the three rounds of IRS conducted) (Kigozi *et al.*, 2012). Thus, knowledge of resistance patterns can have important implications for effective control.

In this study, we assess the insecticide resistance status of field-collected *An. gambiae s.l.* from Jinja, a site of medium-level malaria transmission in which no wide-scale IRS or ITN distribution is currently undertaken.

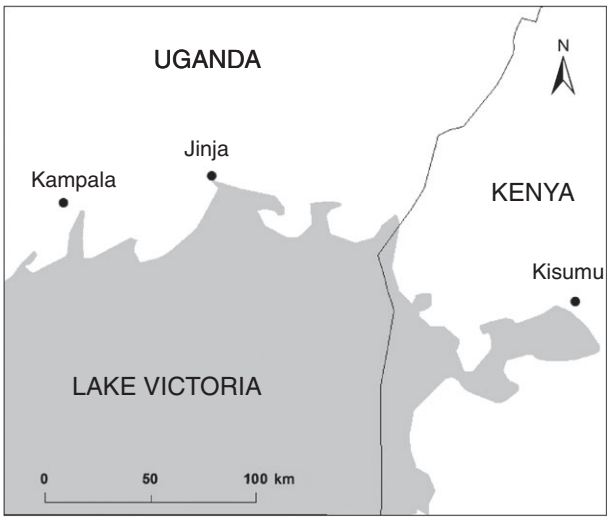

Fig. 1. Map of eastern Uganda depicting the location of Jinja.

Methods

Study site

The study was conducted between the months of July and October, 2011 in Jinja district-Walukuba sub-county (N 00°25.857′ E 033°13.739′) located in Eastern Uganda along the shoreline of Lake Victoria (Fig. 1). Jinja is one of the largest urban centres in Uganda. The region is characterized by hilly grassland and has a bimodal rainfall pattern with a long rainy season between July and November and a short rainy season between February and May. The temperature is relatively constant throughout the year with an average of 25 °C. In the most recent survey, malaria endemicity was characterized as mesoendemic (Okello *et al.*, 2006).

Mosquito sample collections and morphological identification

Mosquitoes were collected as larvae using the dipping method (Service, 1993) from a variety of breeding sites including rice fields, temporary pools along roadsides, tyre tracks and cow hoof prints. Larvae were transferred to the insectary at Walukuba Health Centre, fed on finely ground Tetramin fish food and reared to adulthood. Emerging adults were fed on a 10% sugar solution and identified as belonging to the *An. gambiae* species complex using morphological keys (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987).

Insecticide susceptibility tests

Three- to 5-day-old adult male and non-blood-fed female mosquitoes were exposed to insecticide-treated papers impregnated with WHO diagnostic concentrations (0.05% deltamethrin, 0.75% permethrin, 0.1% bendiocarb, 1% fenitrothion and 4% DDT). Bioassays were conducted in accordance with standard WHO insecticide susceptibility testing procedures

(WHO, 1998). Batches of 20–25 mosquitoes were exposed to each insecticide for 1 h to determine the baseline susceptibility in the population. Secondary investigations were performed to establish time response curves and to characterize the LT_{50} (exposure time required for 50% mortality) to deltamethrin and permethrin through exposure of batches of 20–25 mosquitoes to either deltamethrin or permethrin at different time intervals (1, 5, 15, 30, 45, 60, 90, 120 and 150 min for deltamethrin; 5, 15, 30, 45, 60, 90, 120, 240 and 360 min for permethrin). Apart from the differing exposure times, these tests were in all other ways compliant with the WHO protocol. Temperature and humidity were recorded during both exposure and holding periods, and mortality scored 24 h post exposure. With the exception of mosquitoes that survived exposure beyond 60 min, samples were stored individually over silica gel for further molecular analysis. Mosquitoes that survived an exposure time ≥60 min were killed by aspiration into 90% ethanol, blotted dry and stored immediately in RNALater (Ambion) for future microarray analysis. Legs from these specimens were stored separately for species identification PCR and *kdr Taq*man assays (see below).

For comparison, and to permit generation of resistance ratios (RRs), LT curves were also generated from the pyrethroid susceptible *An. gambiae* Kisumu strain, with exposure times of 0.25, 0.5, 1, 2, 3, 5 and 10 min (deltamethrin) and 0.5, 1, 3, 5, 8, 10 and 15 min (permethrin).

Synergist bioassays

To investigate the possible role of insecticide detoxification by P450 monooxygenases in the resistance phenotype, mosquitoes were exposed for 1 h to papers treated with the synergist piperonyl butoxide (PBO; 4%) prior to exposure to DDT-, deltamethrin- or permethrin-insecticide treated papers for a further 60 min. Mortality was scored after a 24 h recovery period. Controls were exposed to PBO-treated papers then control papers.

Molecular analysis

The species identification PCR of Scott *et al*. (1993) was applied to all samples. *Anopheles gambiae s.s.* molecular form identification (M and S molecular forms) of a subset of samples was completed using the SINE PCR of Santolamazza *et al.* (2008). The *Kdr* (*1014S* and *1014F*) genotype at codon 1014 was determined using the *Taq*man PCR of Bass *et al.* (2007). Exon 21 of the VGSC was sequenced from representative samples using the primers of Lynd *et al*. (2010) to address the issue of introgression of *kdr* alleles from *An. gambiae* to *An. arabiensis* Patton.

Data analysis

Binomial confidence intervals around mortality estimates (Newcombe, 1998) were calculated using Vassar Stats (http://vassarstats.net). LT curves were calculated using a custom R script (R Development Core Team, 2011). Allelic associations with phenotype were investigated using Fisher's Exact tests calculated using Vassar Stats.

Results

Insecticide susceptibility tests and synergist bioassays

A total of 7202 non-blood-fed *An. gambiae s.l.* aged 3–5 days were assayed for resistance using WHO standard 60 min exposures, or for determination of LT curves for permethrin and deltamethrin. In total, 707 *An. gambiae s.s.* and 1536 *An. arabiensis* were exposed to diagnostic doses of deltamethrin (0.05%), permethrin (0.75%), bendiocarb (0.1%), fenitrothion (1%) and DDT (4%) for 1 h. Complete susceptibility to bendiocarb (carbamate) and fenitrothion (organophosphate) was observed in both species and to DDT in *An. arabiensis*. In *An. gambiae s.s.*, resistance was detected to DDT (♀ mortality after 1 h exposure = 48.8 %; ♂ = 29.4%), deltamethrin (♀ 33% and ♂ 49.3%) and permethrin (♀ 24.7% and ♂ 15.5%), and in *An. arabiensis* to permethrin (♀ 56.9% and ♂ 67.1%) with reduced susceptibility to deltamethrin in females only (♀ 84.6 and ♂ 97.5%). Data with 95% confidence intervals are shown in Fig. 2. The possible involvement of cytochrome P450s in the resistance phenotype was investigated using the synergist PBO. Exposure to PBO prior to bioassays was found to fully recover the susceptibility of *An. gambiae s.s.* to deltamethrin, and produce a near full recovery of susceptibility to permethrin, suggesting that P450s are involved in the resistance phenotype. PBO exposure did not alter susceptibility to DDT (Fig. 2). In *An. arabiensis* both permethrin and deltamethrin susceptibility was recovered through prior exposure to PBO.

LT curves for permethrin and deltamethrin are shown in Figs 3 and 4, respectively. We found the LT_{50}s for permethrin in *An. gambiae s.s.* from Jinja to be 207 min (♂) and 132 min (♀). For comparison, the LT_{50}s for the Kisumu strain were 7.8 min (♀) and 5.3 min (♂) giving female and male resistance ratios of 16.9 and 39, respectively. In *An. arabiensis* the LT_{50} for permethrin was 49 min (females) and 33 min (males) with resistance ratios relative to Kisumu of ≈6.2 for both sexes. The LT_{50}s for deltamethrin in *An. gambiae* were higher than those for permethrin (♀ = 319 min; ♂ = 458 min) whereas mortalities for *An. arabiensis* were comparable to the levels for permethrin in this species (♀ = 49 min and ♂ = 42 min) (Fig. 3). For Kisumu, the LT_{50}s for deltamethrin were 1.09 min for females and 0.09 min (95% CIs 0.04–0.24) for males. Because of the extreme susceptibility of males to deltamethrin, the LT_{50} is not reliable and we have utilized the upper confidence limit of the estimate in relative risk (RR) calculations, producing RRs for *An, gambiae* of 292 and 1908 in females and males, respectively, and for *An. arabiensis* RRs of 45 (females) and 175 (males).

Molecular analysis

Only *An. arabiensis* and *An. gambiae s.s.* were detected in Jinja with *An. arabiensis* the more common (70.4%)

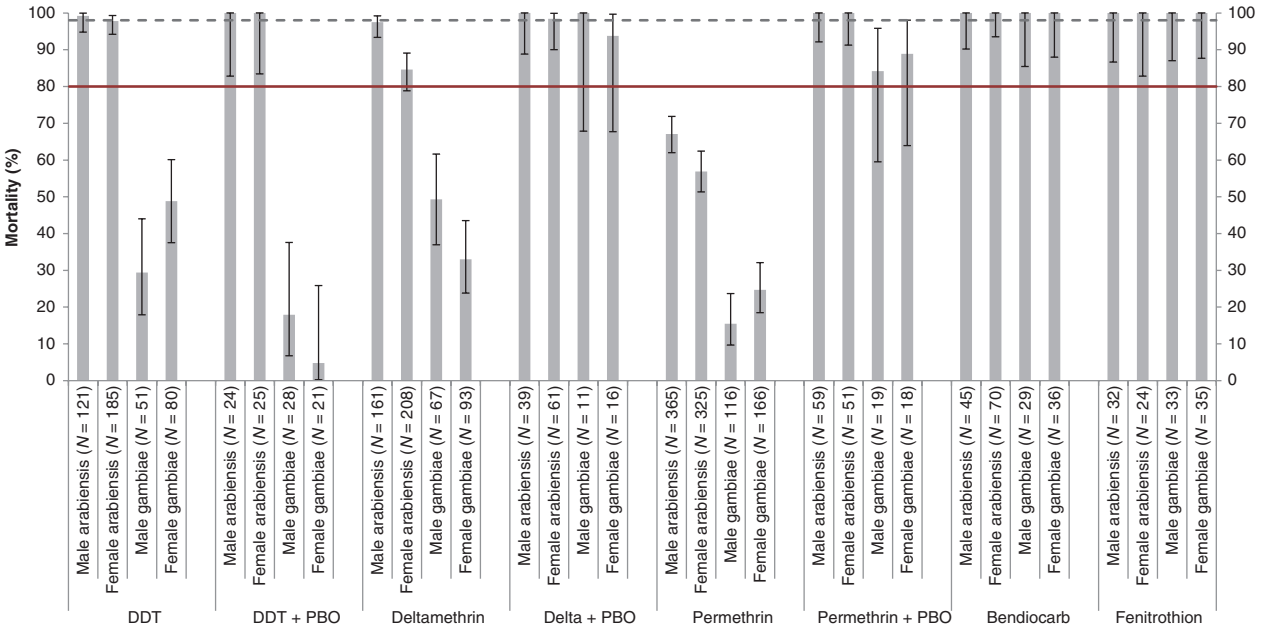

Fig. 2. Mortality levels for *Anopheles gambiae s.s.* and *An. arabiensis* males and females exposed for 1 h to insecticides and the synergist Piperonyl Butoxide (PBO) plus insecticide. The straight line depicts the level of 80% mortality and the dashed line 98% mortality. Mortalities <80% are indicative of resistance under WHO terminology and mortality of 80–98% indicates incipient resistance.

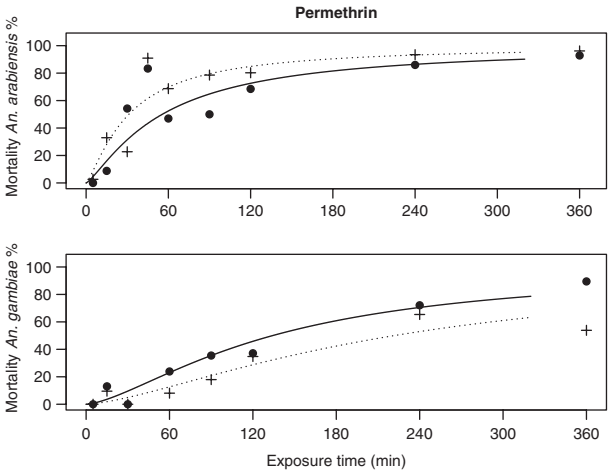

Fig. 3. Species- and sex-specific LT curves for exposure of adult mosquitoes to 0.75% permethrin in a WHO tube bioassay (females: solid lines, black circles; males: dashed lines, crosses). The LT_{50} calculated from these figures are *Anopheles arabiensis* ♀ 48.19 min, *An. arabiensis* ♂ 33.21 min, *An. gambiae* ♀ 135.7 min and *An. gambiae* ♂ 213.3 min.

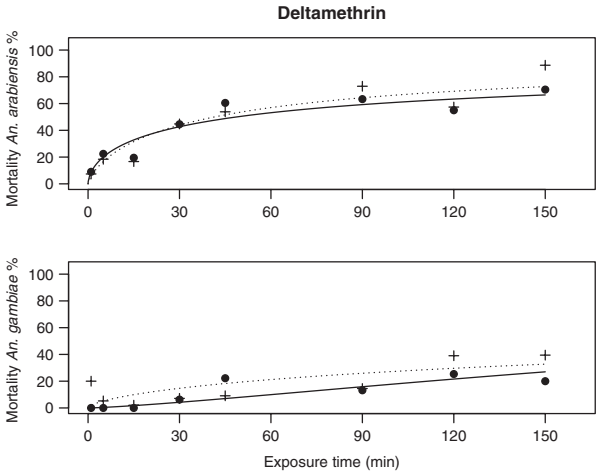

Fig. 4. Species- and sex-specific LT curves for exposure of adult mosquitoes to 0.05% deltamethrin in a WHO tube bioassay. The LT_{50} calculated from these figures are *Anopheles arabiensis* ♀ 48.72 min, *An. arabiensis* ♂ 41.46 min, *An. gambiae* ♀ 319 min and *An. gambiae* ♂ 458 min.

with *An. gambiae* × *An. arabiensis* hybrids detected at a low frequency (0.22%). Only the *An. gambiae* S form were found when a subset of *An. gambiae* ($N = 145$) was analysed with the SINE PCR diagnostic. The *kdr 1014S* allele approached fixation in *An. gambiae s.s.* (95.04%; CI 94.11–95.83%) whereas in *An. arabiensis* only 4 out of 2988 specimens were L/S heterozygotes (frequency of *1014S* 0.07%; CI 0.03–0.18%)—see Table 1. We sequenced *An. arabiensis* heterozygotes (L/S) and L/L homozygotes, and *An. gambiae* homozygous for S/S to determine whether *1014S* might have introgressed from *An. gambiae* to *An. arabiensis.* Sequencing confirmed the presence of the *1014S* allele in *An. arabiensis* but there is insufficient variation in this fragment to discriminate between *de novo* mutation and introgression as the cause. No significant associations between *1014S* and resistance were found for either species phenotyped with any insecticide (alive after exposure to ≥60 min, dead after

Table 1. Genotype counts at codon 1014 of the voltage-gated sodium channel in *Anopheles gambiae* and *An. arabiensis* from Walukuba, Jinja in samples phenotyped for permethrin, deltamethrin and DDT.

			Alive ≥ 60 min				Dead ≤ 60 min				
Insecticide	Sex	Species	LL	LS	SS	FS	LL	LS	SS	FS	*P*
Permethrin	Female	*An. gambiae*	1	14	152	1	0	10	38	0	0.051
Permethrin	Male	*An. gambiae*	3	12	132	0	0	3	22	0	1
DDT	Female	*An. gambiae*	0	4	37	0	0	4	35	0	1
DDT	Male	*An. gambiae*	0	2	34	0	0	1	14	0	1
Deltamethrin	Female	*An. gambiae*	3	5	64	0	0	1	41	0	0.06
Deltamethrin	Male	*An. gambiae*	1	3	36	0	1	3	32	0	1
Permethrin	Female	*An. arabiensis*	177	1	0	0	259	0	0	0	0.41
Permethrin	Male	*An. arabiensis*	116	1	0	0	295	0	0	0	0.28
DDT	Female	*An. arabiensis*	4	0	0	0	166	0	0	0	1
DDT	Male	*An. arabiensis*	1	0	0	0	110	1	0	0	0.4
Deltamethrin	Female	*An. arabiensis*	33	0	0	0	186	0	0	0	1
Deltamethrin	Male	*An. arabiensis*	8	0	0	0	198	0	0	0	1

P-values are from two-tailed exact tests.

exposure to ≤60 min). Although consistent with an hypothesis that *1014S* does not show very strong resistance associations within the population, without genotyping considerably very large sample sizes, the low minor allele frequencies (i.e. commonness of the serine allele in *An. gambiae* and rarity in *An. arabiensis*) curtail the power of the association tests to detect a weak-moderate association with resistance phenotypes.

Through screening 126 *An. arabiensis* and 150 *An. gambiae* for *1014F*, only 1 *An. gambiae* S/F heterozygote was detected and this was confirmed through sequencing (frequency of *1014F* = 0.33% in *An. gambiae*; CI 0.06–1.86%).

Discussion

In this study, we have demonstrated resistance to pyrethroid insecticides in *An. gambiae s.l.* from Jinja, one of the largest population centres in Uganda. For *An. gambiae s.s.*, the resistance levels are particularly high with an LT_{50} for females of over 2 h for permethrin exposure and over 5 h for deltamethrin exposure. Such high levels of resistance are a major concern for malaria control efforts owing to reliance on pyrethroid-impregnated bednets.

Pyrethroid and DDT resistance in Ugandan *An. gambiae s.l.* has been documented previously (Rubaihayo *et al.*, 2008; Ramphul *et al.*, 2009; Verhaeghen *et al.*, 2010). The prevalence of phenotypic resistance appears to have risen sharply in recent years in Jinja. Using standard 1 h WHO diagnostic tests, Verhaeghen *et al.* (2010) observed 74–99% mortality in permethrin-exposed female *An. gambiae* collected between 2004 and 2006. We now observe 25% mortality. No deltamethrin resistance was detected by Verhaeghen *et al.* (2010) although in this study 33% mortality to deltamethrin was detected. Over the same period, DDT mortality was 67–87% and is now 49% (this study). While there are no previous data on resistance for *An. arabiensis* in Jinja, data from Tororo and Busolwe (≈100 km east of Jinja) from 2008 indicate full susceptibility to permethrin and deltamethrin and incipient resistance to DDT in the Busolwe *An. arabiensis* population (Ramphul *et al.*, 2009). Here, we report resistance to permethrin and incipient resistance to deltamethrin in female *An. arabiensis*, suggesting that resistance might be increasing in Uganda. While there is evidence that *An. arabiensis* and *An. gambiae* do not contribute equally to malaria transmission e.g. Okello *et al.* (2006) reported 45% abundance of *An. arabiensis* in Jinja in 2001–2002 but these gave only a 23% contribution to malaria transmission, nevertheless the apparently higher frequency of *An. arabiensis* in this study (70%) than in the 2001–2002 collections of Okello *et al.* (2006) suggests a potentially increased role in overall malaria transmission. Recently, Bayoh *et al.* (2010) documented a species shift in Western Kenya with the gradual replacement of *An. gambiae s.s.* by *An. arabiensis* which was postulated to be in response to increased ITN use. However, ITNs are not used widely in Jinja and hence this is unlikely to explain the apparent rise in *An. arabiensis* frequency in the Jinja population.

In the present study, we failed to detect significant associations between the *kdr 1014S* mutation and any resistance phenotype. However, given the power constraints resulting from low minor allele frequencies in both species (see Weetman *et al.*, 2010) it would be unwise to reject involvement of *1014S* in resistance, especially given previous findings of major involvement of *1014S* in DDT and permethrin resistance in eastern Uganda (Ramphul *et al.*, 2009). Moreover, allele frequency dynamics strongly suggest a strong recent role in resistance. Verhaeghen *et al.* (2010) showed a significant increase of *kdr 1014S* frequency in *An. gambiae s.s* from Jinja, from 13% in 2001/2002 to 34% in 2004/2006. Our data show that the increase in *kdr 1014S* frequency has continued, with the mutation now close to fixation in *An. gambiae s.s.* (≈95%). Such a rapid rate of increase is unlikely to have arisen in the absence of strong DDT or pyrethroid-mediated selection, as implicated elsewhere in cases of *kdr* increase (Protopopoff *et al.*, 2008; Lynd *et al.*, 2010; Mathias *et al.*, 2011). However, *1014S* remains at a very low frequency in *An. arabiensis* (0.07%). We also confirm the finding of Verhaeghen *et al.* (2006) that *1014F* is present in *An. gambiae*

in Uganda, and should now be monitored routinely alongside *1014S*.

The low frequency of *1014S* in *An. arabiensis* suggests that current levels of phenotypic resistance in this species are not related to target site mechanisms and implicate involvement of other factors such as metabolic resistance, cuticular changes or behavioural avoidance. Metabolic resistance to pyrethroids in *An. gambiae* has often been shown to be mediated by cytochrome P450 enzymes (e.g. Müller *et al.*, 2008; Stevenson *et al.*, 2011). The near complete restoration of susceptibility of both species to permethrin and deltamethrin by prior exposure to PBO suggests that P450s are also involved with resistance in the present study. However, this effect was not observed when PBO was used in combination with DDT. While there is evidence that DDT can be metabolized by cytochrome P450s (Chiu *et al.*, 2008; Mitchell *et al.*, 2012), DDT resistance is more commonly associated with metabolism by Glutathione-S-transferases (GSTs) rather than P450s (Chiu *et al.*, 2008) and GSTs are inhibited by DEM (diethyl maleate), which was not employed as a synergist in the present study. Future microarray experiments will aid in understanding the mechanisms underlying the resistance patterns.

We detected a low frequency (0.22%) of hybrids in this population. The presence of hybrids is indicative of the potential for some level of gene flow between the two species (Diabate *et al.*, 2004; Donnelly *et al.*, 2004). *An. gambiae s.s.* × *An. arabiensis* hybrids have also been found in Kenya (Petrarca *et al.*, 1991; Stump *et al.*, 2004) at comparable frequencies (0.2%) and also elsewhere in East Africa (White *et al.*, 1972; Stump *et al.*, 2004). Hybridization between species offers the opportunity for introgression of resistance alleles between species. We detected the *kdr 1014S* allele at a low frequency in *An. arabiensis* and this allele could have arisen *de novo* in *An. arabiensis* (Diabate *et al.*, 2004) or through genetic introgression from *An. gambiae s.s.* (Stump *et al.*, 2004). Unfortunately in the sequence we analysed around the 1014 codon there is insufficient variation to distinguish between these two hypotheses. The presence of *kdr* in *An. gambiae s.s.* populations is believed to threaten malaria control efforts (Vulule *et al.*, 1994; N'Guessan *et al.*, 2007; Rubaihayo *et al.*, 2008; Mathias *et al.*, 2011) hence, the recent appearance of this mutation in *An. arabiensis* indicates that we should assess its impact on malaria control; the very low *kdr* frequency in *An. arabiensis* in this population, presents a valuable opportunity to develop a time series on the frequency of *1014S* in this species for modelling studies (Barbosa *et al.*, 2011).

While resistance to pyrethroids is extremely high in *An. gambiae* and significant in *An. arabiensis*, complete susceptibility to bendiocarb and fenitrothrion was found in both species suggesting a promise for carbamates or organophosphates in future control efforts. A switch from DDT to bendiocarb use in the IRS strategy employed in Apac, northern Uganda, after identification of resistance to DDT and pyrethroids, resulted in a significant drop in malaria cases (Kigozi *et al.*, 2012). The complete susceptibility of the Jinja mosquito population to these insecticides suggests that they are currently likely to be efficacious against both *An. gambiae* and *An. arabiensis*.

Acknowledgements

The project described was supported by Award Number U19AI089674 from the National Institute of Allergy and Infectious Diseases (NIAID). HDM was supported by the Uganda Malaria Clinical Operational and Health Services (COHRE) Training Program at Makerere University, Grant #D43-TW00807701A1, from the Fogarty International Center (FIC) at the National Institutes of Health (NIH). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIAID, FIC or NIH. We thank the staff of Walukuba Health Centre for provision of insectary facilities and the staff of the molecular laboratory (MOLAB) at Makerere University for facilitating access to molecular laboratory facilities.

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Accepted 28 July 2012
First published online 10 October 2012