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Dynamics of insecticide resistance in the malaria vector *Anopheles gambiae s.l.* from an area of extensive cotton cultivation in Northern Cameroon

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Summary

OBJECTIVE To explore temporal variation in insecticide susceptibility of *Anopheles gambiae s.l.* populations to the four chemical groups of insecticides used in public health and agriculture, in close match with the large-scale cotton spraying programme implemented in the cotton-growing area of North Cameroon.

METHODS Mosquito larvae were collected in 2005 before (mid June), during (mid August) and at the end (early October) of the cotton spraying programme. Larvae were sampled in breeding sites located within the cotton fields in Gaschiga and Pitoa, and in Garoua, an urban cotton-free area that served as a control. Insecticide susceptibility tests were carried out with 4% DDT (organochlorine), 0.4% chlorpyrifos methyl (organophosphate), 0.1% propoxur (carbamate), 0.05% deltamethrin and 0.75% permethrin (pyrethroids).

RESULTS Throughout the survey, *An. gambiae s.l.* populations were completely susceptible to carbamate and organophosphate, whereas a significant decrease of susceptibility to organochlorine and pyrethroids was observed during spraying in cotton-growing areas. Tolerance to these insecticides was associated with a slight increase of knockdown times compared to the reference strain. Among survivor mosquitoes, the East and West African *Kdr* mutations were detected only in two specimens of *An. gambiae s.s.* (n = 45) and not in *Anopheles arabiensis* (n = 150), suggesting metabolic-based resistance mechanisms.

CONCLUSIONS Environmental disturbance due to the use of insecticides in agriculture may provide local mosquito populations with the enzymatic arsenal selecting tolerance to insecticides.

keywords malaria, *Anopheles gambiae*, *Anopheles arabiensis*, insecticide resistance, agriculture, Cameroon

Introduction

Insecticide resistance in malaria vectors has become a major concern for public health authorities and national malaria control programmes in Africa, where the prevention of this highly devastating disease heavily relies on the use of pesticides for the control of its mosquito vector populations (WHO 2002). Several studies have demonstrated the efficacy of both insecticide treated nets (ITNs) and indoor residual spraying (IRS) for curbing malaria incidence (Curtis & Mnzanva 2000; Guyatt *et al.* 2002). Today, 12 insecticides belonging to four chemical groups are available for IRS (six pyrethroids, three organophosphates, two carbamates and the highly debated organochlorine DDT), but only pyrethroids have been approved for impregnating bednets, mainly because of their low toxicity for humans and speed of killing compared to other pesticides (Zaim *et al.* 2000; Najera & Zaim 2001; WHO 2004, 2006). However, resistance to all these compounds has been described in insect vectors of human diseases, including African malaria vectors (WHO 1970; Hemingway & Ranson 2000). The ongoing spread of

insecticide-resistant genes, such as the well-characterized *Kdr* mutations (Martinez-Torres *et al.* 1998; Ranson *et al.* 2000a) in populations of the major African malaria vectors *Anopheles gambiae* and its sibling species *Anopheles arabiensis* (Chandre *et al.* 1999; Diabaté *et al.* 2004; Etang *et al.* 2006; Tripet *et al.* 2007), can seriously jeopardize the efficacy of vector control programmes. This was recently demonstrated in Benin (N'Guessan *et al.* 2007) whereas in South Africa, pyrethroid resistance in *Anopheles funestus* necessitated a switch back to the use of DDT for IRS so as to restore the efficacy of the control programme (Hargreaves *et al.* 2000).

The basic mechanisms underlying insecticide resistance include insecticide target-site mutations in structural genes of the central nervous system of the insect such as GABA-receptors, acetylcholinesterase and sodium channels, and increased metabolic detoxification of the insecticide through elevated carboxylesterases, cytochrome P450 oxidases or glutathione transferases enzymatic activities (Hemingway & Ranson 2000; Hemingway *et al.* 2004; Li *et al.* 2007). Biochemical and molecular techniques have been developed for high-throughput resistant-gene frequency estimation and monitoring in natural vector populations, allowing detection of resistance at an early stage. Availability of the genome sequence of *An. gambiae* (Holt *et al.* 2002) and the subsequent development of specific genomic tools such as the *Detox* genechip (David *et al.* 2005) improve our ability to unravel the complexity of the networks of genes involved in insecticide resistance and their regulatory pathways. Such knowledge is of paramount importance for establishing rational strategies for insecticide resistance management to preserve the efficacy of the limited number of pesticides available for malaria control. Moreover, in addition to an in-depth knowledge of the biology and genetics of the vector(s) involved, insecticide resistance management requires a clear understanding of the environmental factors that favour the rise in frequency and further spread of such selectively advantageous alleles.

All the insecticides currently recommended for IRS or ITNs were initially developed for pest management in agriculture and they are used as agrochemicals and for animal health in far greater proportions than they are for public health purposes (Zaim & Guillet 2002). This past and present widespread use of pesticides for agricultural purposes is supposed to have favoured the emergence and facilitated the spread of resistance within and between mosquito populations (Georghiou *et al.* 1973; Penilla *et al.* 1998; Chandre *et al.* 1999; Guillet *et al.* 2001; Fanello *et al.* 2003). Whether this side effect is common or exceptional has long raised controversy (Lines 1988), and the role of agricultural and public health use of insecticides in the evolution of resistance in diseases vectors is still an open question. Consistent with a strong selective pressure exerted by agricultural use of insecticides, a number of studies reported high-level resistance to pyrethroids and DDT associated with high frequencies of the *Kdr* mutations in populations of *An. gambiae* from cotton fields in West Africa (Diabaté *et al.* 2002, 2004; Tripet *et al.* 2007). Other studies provided evidence for selection of *Kdr* alleles associated with the use of ITNs and other domestic use of pyrethroids for personal protection (Kolaczinski *et al.* 2000; Pinto *et al.* 2006), especially where large-scale pilot interventions have been implemented (Stump *et al.* 2004). The same probably applies to metabolic-based resistance mechanisms which, although less tractable, may be more important than, and as widespread as *Kdr*-based resistance (Penilla *et al.* 1998; Hemingway & Ranson 2000; Etang *et al.* 2003, 2007).

In this study, we monitored the level of resistance of *An. gambiae s.l.* populations to the four chemical groups of insecticides used in public health and agriculture in an area of extensive cotton cultivation in North Cameroon. Using bioassays, we explored temporal variation in insecticide susceptibility in close match with the large-scale cotton spraying programme implemented in the area. We provide evidence for temporal fluctuation of the levels of resistance to different compounds in the resident mosquito populations and draw insights into the resistance mechanisms involved and putative selective pressure exerted on these mosquito populations.

Materials and methods

Study area and cotton crops spraying programme

The study was conducted in an area of extensive cotton cultivation in Northern Cameroon (35 000 ha cultivated in 2005, Figure 1), where malaria is endemic and seasonal (Chouaibou *et al.* 2006). The climate is typical of the Soudanian tropical region, with 700–1000 mm annual rainfall over 6 months (May–October). Mosquitoes were sampled in three localities 5–10 km apart within the area: Garoua, Gaschiga and Pitoa. Garoua is an urban, cotton-free area and was used as a control, while Gaschiga and Pitoa are rural areas surrounded by cotton fields. Spraying of cotton crops in Gaschiga and Pitoa occurs fortnightly between July and October, according to a well-established calendar implemented and monitored by the Société de Développement du Coton (SODECOTON). In 2005, a cyclodiene organochlorine (endosulfan 500 g/l; 750 ml/ha) was used for the first two rounds of treatments (July, 15–August, 11), then a mixture of pyrethroid (cypermethrin 200 g/l; 180 ml/ha) and organophosphate

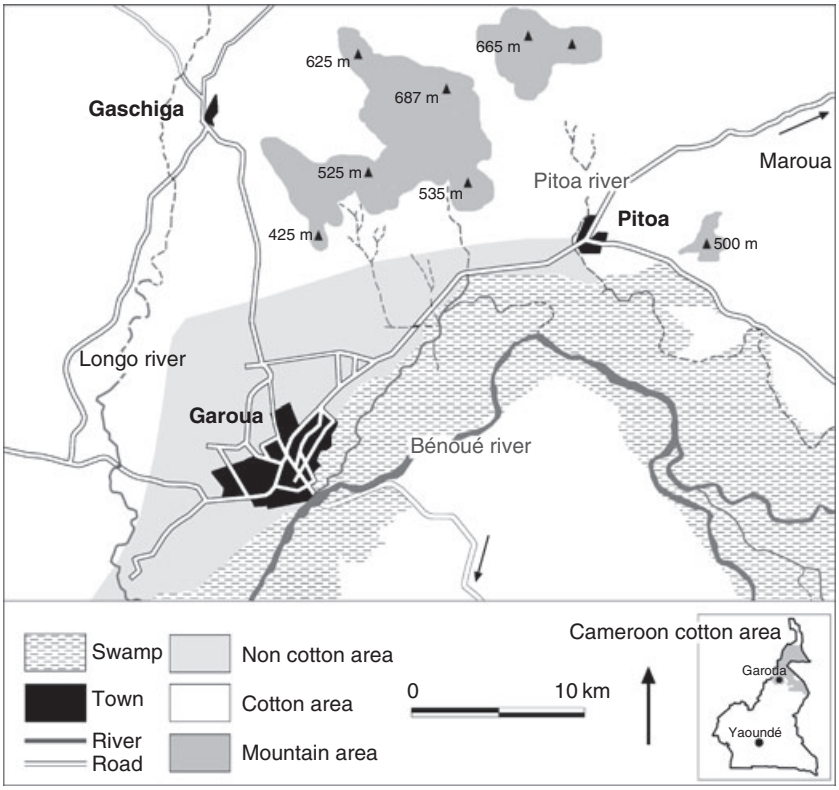

Figure 1 Map of the study area showing the three sampling sites and their localisation with respect to cultivated cotton fields.

(profenofos 500 g/l, 300 ml/ha) insecticides (Pyr/OP) was used until the end of the spraying programme (August, 12–October, 20), except between September 9 and 22, when endosulfan was applied again (500 g/l; 1 l/ha).

Mosquito sampling

During the 2005 rainy season, immature stages of *An. gambiae* were collected from breeding sites simultaneously in the three localities, three times: before the onset of cotton treatments (mid June), at the end of endosulfan–onset of Pyr/OP treatment (mid August), and at the end of the spraying programme (early October). In Pitoa and Gaschiga, larvae were collected from breeding sites within the cotton fields. Larvae from breeding sites sampled in a given locality were pooled and allowed to emerge locally. Emerging adult mosquitoes were used for insecticide susceptibility tests.

Insecticides susceptibility tests

WHO insecticide susceptibility test-kits and standard procedures (WHO 1998) were used to monitor the susceptibility of wild *An. gambiae* populations to technical material of the four chemical groups of insecticides commonly used in public health and agriculture. Batches of 20 unfed *An. gambiae s.l.* females 2–4 days old were exposed to filter papers impregnated with 4% DDT (organochlorine), 0.4% chlorpyrifos methyl (organophosphate), 0.1% propoxur (carbamate), 0.05% deltamethrin and 0.75% permethrin (pyrethroids). Impregnated papers were obtained from the WHO reference center at the Vector Control Research Unit, University Sains Malaysia (Penang, Malaysia). For each test session, 40–100 mosquitoes (two to five batches of 20 mosquitoes) were exposed to untreated filter papers to serve as controls and 79–100 mosquitoes (four to five batches of 20 mosquitoes) were used against each insecticide to be tested. All the bioassays were conducted in the Pitoa field station, at a temperature of 25–27 °C.

Insecticide knockdown effects were monitored throughout the 1 h-exposure to insecticide impregnated papers, and mortality was scored 24 h post-exposure. The *An. gambiae* Kisumu strain was used as the reference susceptible strain and tested simultaneously to the field populations. All specimens used for bioassays were then stored individually in numbered tubes with desiccant and preserved at −20 °C until laboratory processing.

Molecular identification and *kdr* genotyping

Because bioassays were conducted on adults emerging from larvae collected directly in the fields, molecular identification of the specimens could only be processed *a posteriori*. A sample of 40 *An. gambiae s.l.* specimens was drawn at random from the pool of unexposed mosquitoes (bioassays controls) per site and per time point and was considered as representative of the mosquito population being tested. Individual mosquitoes were identified down to their species and molecular form with the PCR-RFLP method described by Fanello *et al.* (2002), which allows simultaneous identification of the M and S molecular forms within *An. gambiae s.s.*, as well as the sibling species *An. arabiensis*. Mosquitoes that survived insecticide susceptibility tests were identified in the same way.

Detection of *Kdr* mutations was performed on all mosquitoes that survived exposure to the insecticides, using a recently described hot oligonucleotide ligation assay (HOLA), a method developed to detect both the East and West-African *Kdr* alleles in a homozygous or heterozygous state (Lynd *et al.* 2005).

Data analysis

WHO (1998) criteria were used to evaluate the resistance/susceptibility status of the tested mosquito populations. By the said criteria, resistance is indicated by mortality rates below 80% while mortality rates greater than 98% are indicative of susceptibility. Mortality rates between 80–98% suggest increased tolerance, but resistance should be confirmed.

Log-probit analyses were used to model and compare the relationship between time of exposure and knockdown rates for DDT and pyrethroids (OPs and carbamates do not induce any knockdown effect) using WINDL software (Giner *et al.* 1999). The time of exposure causing 50% and 95% knockdown in the tested population (KdT_{50} and KdT_{95} respectively) was derived from expected values for DDT and pyrethroids in each locality and per time point.

Results

Susceptibility to insecticides

The *An. gambiae* Kisumu reference strain was susceptible to all insecticides, showing 100% mortality at WHO-recommended discriminating dosages (Figure 2). Mortality in control groups was consistently below 5%, and no correction was required.

Throughout the survey, *An. gambiae s.l.* populations were completely susceptible to carbamate and organophosphate insecticides in the three localities (mortality: 99–100%), whereas their susceptibility to DDT and pyrethroids fell, especially in cotton-growing areas (Figure 2).

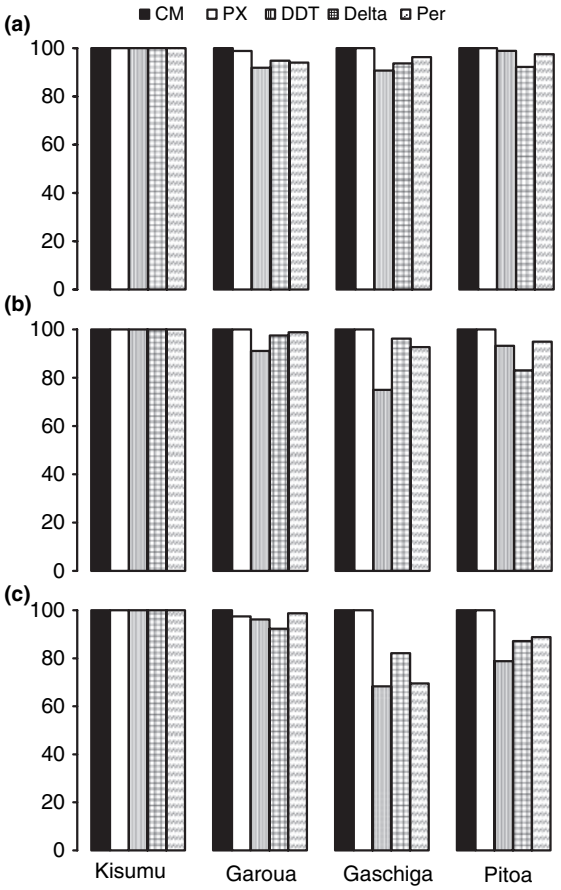

Figure 2 Mortality rates 24 h post-exposure to insecticides in *Anopheles gambiae s.l.* populations from North Cameroon. (a) June 2005, prior to the implementation of cotton spraying programme, (b) August 2005, end of endosulfan–onset of Pyr/OP treatments, (c) October 2005, end of the cotton treatments. CM, 0.4% chlorpyrifos methyl; PX, 0.1% propoxur; DDT, 4% DDT; Delta, 0.05% deltamethrin; Per, 0.75% permethrin; Kisumu, *An. gambiae* susceptible reference strain (Kisumu).

In June, before the onset of cotton treatments, mosquitoes collected in both cotton-growing (Gaschiga and Pitoa) and cotton-free (Garoua) areas showed 91–98% mortality to DDT and pyrethroids, suggesting low-level resistance to these compounds (Figure 2a). In August, during cotton crop treatments (end of endosulfan–onset of Pyr/OP treatment), resistance levels to DDT and pyrethroids had increased in the cotton-growing areas but not in the cotton-free area (Figure 2b). At the end of the cotton spraying programme (October), resistance levels to DDT and

Table 1 Knockdown times (KdT_{50} and KdT_{95}) in minutes for *Anopheles gambiae s.l.* populations in North Cameroon

Time point	Locality	Insecticide	KdT_{50} [CI] (min)	KdT_{95} [CI] (min)	$KdT_{50}R$
June 2005	Garoua	4% DDT	49.08 [47.71–50.63]	83.44 [77.76–91.07]	1.82
		0.05% Deltamethrin	22.60 [21.65–23.53]	51.74 [48.73–55.39]	1.57
		0.75% Permethrin	15.90 [15.10–16.68]	38.19 [35.95–40.87]	1.21
	Gaschiga	4% DDT	43.16 [42.20–44.16]	69.48 [66.28–73.46]	1.59
		0.05% Deltamethrin	24.25 [23.41–25.06]	45.67 [43.56–48.20]	1.69
		0.75% Permethrin	11.67 [10.52–12.76]	56.90 [51.09–64.62]	0.88
	Pitoa	4% DDT	38.09 [37.09–39.07]	66.68 [63.48–70.67]	1.41
		0.05% Deltamethrin	13.41 [12.67–14.13]	31.41 [29.50–33.73]	0.93
		0.75% Permethrin	12.59 [11.61–13.54]	45.00 [41.44–49.46]	0.95
August 2005	Garoua	4% DDT	41.81 [40.78–43.06]	74.66 [70.37–80.12]	1.55
		0.05% Deltamethrin	15.17 [14.38–15.94]	35.85 [33.72–38.43]	1.06
		0.75% Permethrin	19.22 [18.45–19.96]	36.95 [35.12–39.15]	1.45
	Gaschiga	4% DDT	45.72 [44.52–47.00]	78.22 [73.38–84.63]	1.69
		0.05% Deltamethrin	24.19 [23.35–25.00]	42.21 [40.38–44.42]	1.68
		0.75% Permethrin	18.35 [17.46–19.18]	33.55 [31.92–35.55]	1.38
	Pitoa	4% DDT	38.14 [37.20–39.08]	62.71 [59.93–66.11]	1.41
		0.05% Deltamethrin	21.03 [20.27–21.76]	35.20 [33.69–37.03]	1.41
		0.75% Permethrin	18.15 [17.19–19.06]	37.93 [35.88–40.42]	1.37
October 2005	Garoua	4% DDT	37.71 [36.68–38.78]	64.30 [61.19–68.20]	1.39
		0.05% Deltamethrin	22.78 [20.08–25.16]	74.06 [65.05–88.09]	1.59
		0.75% Permethrin	14.18 [13.44–14.87]	26.21 [24.77–27.99]	1.07
	Gaschiga	4% DDT	47.15 [45.68–48.79]	89.08 [81.88–99.15]	1.75
		0.05% Deltamethrin	20.29 [19.40–21.07]	40.69 [38.70–43.08]	1.42
		0.75% Permethrin	15.48 [14.77–16.17]	32.05 [30.31–34.14]	1.17
	Pitoa	4% DDT	42.44 [41.01–43.91]	82.85 [76.45–89.66]	1.57
		0.05% Deltamethrin	22.23 [20.88–23.51]	61.17 [56.70–66.89]	1.55
		0.75% Permethrin	16.57 [15.62–17.45]	32.13 [30.43–34.21]	1.25

$KdT_{50}R$, KdT_{50} of the tested population divided by KdT_{50} of the Kisumu reference strain.

pyrethroids had increased again in cotton-growing areas (Figure 2c). At this time, mosquitoes were resistant to DDT and permethrin in Gaschiga (mortality below 70% to both insecticides) and to DDT in Pitoa. This trend for increased resistance throughout the 2005 rainy season was statistically significant for DDT and permethrin in Gaschiga (Mantel-Haensel stratified test on mortality data, $\chi^2 = 11.4$, $P < 0.001$) and for DDT in Pitoa ($\chi^2 = 9.0$, $P < 0.05$).

Resistance to DDT and pyrethroids was associated with a slight increase of knockdown times (Table 1). However, the ratios of KdT_{50} compared to the reference strain ($KdT_{50}R$ in Table 1) were below 1.82, suggesting low evidence for *Kdr*-related resistance mechanism.

Species identification within the *An. gambiae* s.l. complex

Molecular identification of sibling species within the *An. gambiae* complex showed that the great majority of the specimens tested were *An. arabiensis* (276/360 = 77%), occurring together with the S molecular form of *An. gambiae s.s.* (84/360 = 23%). Both species were found in all three localities and at each time point (Figure 3). However, the relative frequency of the two species varied greatly in space and time, with a relative frequency of *An. arabiensis* ranging from 55% in Gaschiga in August, to 97.5% in Pitoa in June. Significant differences in the relative frequencies of both species were detected over time in Garoua ($\chi^2 = 23.5$, $P < 0.001$) and Pitoa ($\chi^2 = 14.9$, $P < 0.001$) but not in Gaschiga ($\chi^2 = 5.7$, $P > 0.05$), and between the three localities in June ($\chi^2 = 18.5$, $P < 0.001$) and August ($\chi^2 = 17.1$, $P < 0.001$) but not in October ($\chi^2 = 2.6$, $P > 0.05$).

To further explore whether both *An. arabiensis* and *An. gambiae* were able to survive exposure to insecticides, and to assess whether temporal variation in resistance could be linked to the changing species composition within each population, mosquitoes that survived exposure to insecticides were identified to species. Among the 195 surviving mosquitoes that were successfully identified by PCR-RFLP, 150 (77%) were *An. arabiensis* and 45 (23%) were *An. gambiae*-S form. Table 2 provides further insights into the levels of resistance to DDT and pyrethroids of both species in Pitoa and Gaschiga, when raw bioassays data

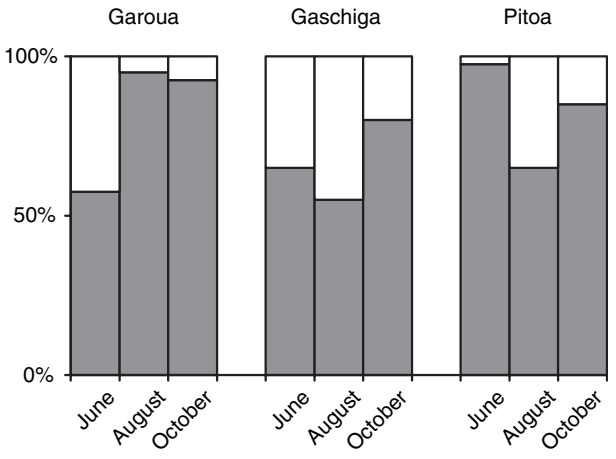

Figure 3 Relative frequencies of *Anopheles arabiensis* (grey) and *Anopheles gambiae*-S form (white) in larval collections from three localities in North Cameroon across the 2005 rainy season. For each locality and sampling date, molecular identifications were conducted on 40 specimens randomly selected from the pools of mosquitoes used for bioassays controls.

suggested significant decrease in susceptibility to these compounds (see above). Interestingly, both *An. arabiensis* and *An. gambiae*–S form from Gaschiga and Pitoa were resistant to DDT but only the *An. arabiensis* population from Gaschiga also showed resistance to both pyrethroids tested. The *An. gambiae*-S form population from Pitoa also showed resistance to deltamethrin at the end of the survey, although low sample size (estimated *An. gambiae*–S form sample size = 12 specimens) precludes any firm conclusion in this case.

Kdr genotyping

Among the 195 mosquitoes that survived insecticide exposure during the bioassays, the East and West African *Kdr* mutations were detected in two different specimens of *An. gambiae s.s.* (n = 45), at the heterozygous state. All the specimens of *An. arabiensis* tested (n = 150) had the susceptible genotype (TTA/TTA).

Discussion

This study demonstrates temporal variation in susceptibility of *An. gambiae s.l.* populations to different classes of insecticides commonly used in public health and agriculture, in an area of extensive cotton cultivation in northern Cameroon. Seasonal variation in insecticide susceptibility is important to acknowledge because it can seriously bias the outcome of transversal susceptibility assays that are to be conducted prior to the implementation of any large-scale malaria vector control initiative (WHO 2006), and could thus lead to erroneous choices of insecticides.

In the rice fields of Burkina Faso, temporal shifts in resistance levels observed at the end of the rainy season, when temporary breeding sites dry up, were linked to the seasonal immigration in the area of *Kdr*-resistant *An. gambiae*-S form mosquitoes originating from the neighbouring cotton fields, taking over the susceptible M form populations (Diabaté *et al.* 2002). Temporal variation in species composition within the *An. gambiae s.l.* complex was also observed in our study sites. In agreement with former findings from the area (Etang *et al.* 2003, 2007; Wondji *et al.* 2005; Chouaibou *et al.* 2006), the natural populations of *An. gambiae s.l.* were mainly composed of *An. arabiensis*, occurring together with *An. gambiae s.s.* of the S molecular form. Increased tolerance to DDT was observed in both species, but cross-resistance to pyrethroids seemed to occur in *An. arabiensis* only. Such pattern might result from fine-scale physiological or behavioural differences between species in the area, rendering *An. arabiensis* more extensively exposed to selective pressure from pyrethroids than its sibling species, or to the selection of alternative resistance mechanisms in the different species. Indeed, although occurring at very low frequency, both *Kdr* mutations were detected in the *An. gambiae*-S form population from the area and not in *An. arabiensis*. This situation prompts careful monitoring because the known quick spread of these mutations within and between *An. gambiae* populations could result in a subsequent increase of resistance levels in the coming years, with a great impact on the efficacy of insecticide-based vector control (N'Guessan *et al.* 2007).

Prior to the implementation of a season-wide spraying programme directed against cotton pests, reduced susceptibility to DDT and pyrethroids (deltamethrin and permethrin) was observed in all three localities sampled. Bioassays conducted in the area in 1997–2000 already detected reduced susceptibility to deltamethrin in Pitoa, with a mortality rate of 88.7% that falls within the range observed in the present study (83.1–92.2%), but populations of *An. gambiae s.l.* from the entire area were fully susceptible to DDT (mortality rate = 100%, Etang *et al.* 2003). Our findings of baseline tolerance to DDT in all populations is of major concern as this insecticide has been selected by the National Malaria Control Program of Cameroon for the forthcoming implementation of a country-wide IRS programme (E. Fondjo, personal communication).

Furthermore, levels of resistance to DDT and, to a lower extent to pyrethroids, steadily increased throughout the 2005 rainy season. This was observed in populations of

Table 2 Breaking down resistance phenotype to DDT and pyrethroids by species in the *Anopheles gambiae s.l.* populations from Gaschiga and Pitoa

		DDT				Deltamethrin				Permethrin			
	Control	No. tested*	No. survivors	% Mortality	Status	No. tested*	No. survivors	% Mortality	Status	No. tested*	No. survivors	% Mortality	Status
Gaschiga													
August 2005													
Anopheles gambiae s.l.	40	80	20	75.0	R	79	3	96.2	RC	82	6	92.7	RC
Anopheles arabiensis	22	44	11	75.0	R	43	3	93.1	RC	45	5	88.9	RC
Anopheles gambiae-S form	18	36	9	75.0	R	36	0	100.0	S	37	1	97.3	RC
October 2005													
An. gambiae s.l.	40	79	25	68.4	R	84	15	82.1	RC	83	25	69.9	R
An. arabiensis	31	61	14	77.1	R	65	15	77.0	R	64	25	61.1	R
An. gambiae-S form	9	18	11	38.1	R	19	0	100.0	S	19	0	100.0	S
Pitoa													
August 2005													
An. gambiae s.l.	40	77	5	93.5	RC	83	14	83.1	RC	78	4	94.9	RC
An. arabiensis	26	50	3	94.0	RC	54	10	81.5	RC	51	4	92.1	RC
An. gambiae-S form	14	27	2	92.6	RC	29	4	86.2	RC	27	0	100.0	S
October 2005													
An. gambiae s.l.	40	73	19	74.0	R	78	10	87.2	RC	80	9	88.8	RC
An. arabiensis	34	62	16	74.2	R	66	7	89.4	RC	68	9	86.8	RC
An. gambiae-S form	6	11	3	72.6	R	12	3	74.4	R	12	0	100.0	S

*Sample sizes per species were extrapolated from diagnostic PCR data obtained from the control sample.

Status: resistance status were determined according to WHO (1998) guidelines: R, resistance (mortality below 80%); S, susceptibility (mortality above 98%); RC, resistance to be confirmed (mortality above 80% and below 98%).

mosquitoes collected within the area exposed to a putative selective pressure exerted by the cotton spraying programme but not in a neighbouring urban setting, outside of cotton fields. Such patterns might suggest some influence of the insecticides used for cotton spraying on reducing susceptibility of the resident *An. gambiae* populations to these classes of insecticides, as reported by Diabaté *et al.* (2002) in Burkina Faso. Similar seasonal escalation of resistance to different classes of insecticides closely matching insecticide use for cotton spraying was also reported for *An. albimanus* in El Salvador (Georghiou *et al.* 1973).

However, the pattern of resistance we observed in our study area poorly supports this view. Indeed, no use of DDT is reported in Cameroon since its ban in the 1960s, neither for agricultural nor for public health purposes and unofficial use of this or related compounds is unlikely in our study area, due to strong enforcement of insecticide usage policies within the SODECOTON area. Traces or residues of DDT might still be found in the environment, but these could not easily explain the seasonal rise in resistance levels we document in the present study. Cross-resistance between DDT and pyrethroids has been extensively demonstrated, due to the *Kdr* point mutations in the common target site of these insecticides, the voltage-gated sodium channel (Martinez-Torres *et al.* 1998; Chandre *et al.* 1999; Ranson *et al.* 2000a).

Wherever they are present, the *Kdr* mutations might be selected by either DDT or pyrethroids and will provide cross-resistance to all related compounds. High-level resistance to DDT and pyrethroids associated to high frequency of the *Kdr* mutations might have originally been selected for by the extensive use of DDT for agriculture and/or large scale experimental malaria vector control programmes conducted in the 1950s, and subsequently, by the increased use of pyrethroids for the same purposes and for personal protection (Elissa *et al.* 1993; Chandre *et al.* 1999; Diabaté *et al.* 2002; Pinto *et al.* 2006; Tripet *et al.* 2007). In our study area however, analysis of knockdown times and the absence of *Kdr* mutations in *An. arabiensis* clearly ruled out this widespread insecticide resistance mechanism as a cause of the resistance phenotype we observed, and suggest alternative, metabolic-based resistance mechanisms, as formerly observed in the area (Etang *et al.* 2003, 2007).

In Cameroon, pyrethroids have been widely used on cotton for approximately 20 years because of their efficacy to control a wide range of pests at a low cost. In the course of the 2004 cotton growing season, several pest control failures in farmers' fields were attributed to pyrethroid resistance in the cotton bollworm, *Helicoverpa armigera* (Brévault & Achaleke 2005). As previously reported in West Africa (Martin *et al.* 2002), metabolic resistance to pyrethroids via the overproduction of cytochrome P450 and/or non-specific esterases (NSEs) seemed to be the major resistance mechanisms (Achaleke J & Brevault T, unpublished observation). Moreover, elevated levels of NSEs and mixed function oxidases were recently described in the *An. gambiae s.l.* population of Pitoa sampled in 1999–2001 (Etang *et al.* 2007). Biochemical assays and specific gene expression studies are currently under way to further explore this hypothesis and to more broadly characterize the genetic basis and regulatory pathways responsible for insecticide resistance in *An. arabiensis* populations from this area.

Cross-resistance between DDT and pyrethroids is difficult to explain in the absence of *Kdr* mutations, because of the high substrate specificity and selectivity of the enzymes involved in insecticide detoxification pathways (Hayes & Pulford 1995; Hemingway & Karunaratne 1998; Ranson *et al.* 2000b; McAbee *et al.* 2003; Huang *et al.* 2005). For the same reason, it is hard to imagine that the cocktail of insecticides used during cotton treatment, which includes a cyclodiene (endosulfan), an OP (profenofos) and a pyrethroid (cypermethrin) might have selected specific detoxification mechanisms to unrelated compounds such as DDT. However, these insecticides, alone or in combination with other man-made pollutants and natural xenobiotics (such as natural plant toxins, herbicides or heavy metals released in the environment by human activities) might expose larval mosquito populations to strong chemical disturbance and select for general mechanisms for increased ability to control oxidative stress and other broad metabolic disorders, with a putative side effect on tolerance to insecticides (Boyer *et al.* 2006; David *et al.* 2006; Li *et al.* 2007).

Indeed, besides their neurotoxic effect, pyrethroids and other insecticides induce oxidative stress and disturbance of antioxidant pathways in insects and mammals (Vontas *et al.* 2001; Abdollahi *et al.* 2004). Furthermore, a number of enzymes and effectors involved in oxidative stress reduction and the metabolism of reactive oxygen species are constitutively over-expressed in DDT- and/or pyrethroids-resistant strains of *An. gambiae* (Ding *et al.* 2003; David *et al.* 2005; Vontas *et al.* 2005; Müller *et al.* 2007) and other insect species (Vontas *et al.* 2001; Pedra *et al.* 2004). Constitutive over-expression of such antioxidant molecules induced by the release in the environment of a number of pollutants and xenobiotics might therefore provide mosquito populations with the enzymatic arsenal allowing baseline tolerance to unrelated insecticide compounds through an increased resistance to the oxidative stress they induce. Such indirect effect of global landscape

pollution and chemical disturbances on the ability of mosquito populations to resist insecticides of public health interest has not been adequately studied and deserves further attention because it can potentially impact the kinetics of emergence and the nature of the resistance mechanisms that might be selected in malaria vectors breeding in xenobiotics-rich (e.g. densely populated urban areas, industrial and agro-industrial landscapes) and xenobiotics-poor (e.g. remote rural and sylvan) environments.

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References

Abdollahi M, Ranjbar A, Shadnia S *et al.* (2004) Pesticides and oxidative stress: a review. *Medical Science Monitor* **10**, 141–147.

Boyer S, Sérandour J, Lempérière G *et al.* (2006) Do herbicide treatments reduce the sensitivity of mosquito larvae to insecticides? *Chemosphere* **65**, 721–724.

Brévault T & Achaleke J (2005) Status of pyrethroid resistance in the cotton bollworm, *Helicoverpa armigera*, in Cameroon. *Resistant Pest Management Newsletter* **15**, 4–7.

Chandre F, Darriet F, Manga L *et al.* (1999) Status of pyrethroid resistance in *Anopheles gambiae s.l. Bulletin of the World Health Organization* **77**, 230–234.

Chouaibou M, Simard F, Chandre F *et al.* (2006) Efficacy of bifenthrin-impregnated bednets against *Anopheles funestus* and pyrethroid-resistant *Anopheles gambiae* in North Cameroon. *Malaria Journal* **5**, 77.

Curtis FC & Mnzanva AEP (2000) Comparison of house spraying and insecticide-treated nets for malaria control. *Bulletin of the World Health Organization* **78**, 1389–1400.

David JP, Strode C, Vontas J *et al.* (2005) The *Anopheles gambiae* detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 4080–4084.

David JP, Boyer S, Mesneau A *et al.* (2006) Involvement of cytochrome P450 monooxygenases in the response of mosquito larvae to dietary plant xenobiotics. *Insect Biochemistry and Molecular Biology* **36**, 410–420.

Diabaté A, Baldet T, Chandre F *et al.* (2002) The role of agricultural use of insecticides in resistance to pyrethroids in *Anopheles gambiae s.l.* in Burkina Faso. *American Journal of Tropical Medicine and Hygiene* **67**, 617–622.

Diabaté A, Brengues C, Baldet T *et al.* (2004) The spread of the Leu-Phe kdr mutation through *Anopheles gambiae* complex in Burkina Faso: genetic introgression and de novo phenomena. *Tropical Medicine and International Health* **9**, 1267–1273.

Ding Y, Ortelli F, Rossiter LC *et al.* (2003) The *Anopheles gambiae* glutathione transferase supergene family: annotation, phylogeny and expression profiles. *BMC Genomics* **4**, 35.

Elissa N, Mouchet J, Rivière F *et al.* (1993) Resistance of *Anopheles gambiae s.s.* to pyrethroids in Côte d'Ivoire. *Annales de la Société Belge de Médecine Tropicale* **73**, 291–294.

Etang J, Manga L, Chandre F *et al.* (2003) Insecticide susceptibility status of *Anopheles gambiae s.l*(Diptera: Culicidae) in the republic of Cameroon. *Journal of Medical Entomology* **40**, 491–497.

Etang J, Fondjo E, Chandre F *et al.* (2006) Short report: first report of knockdown mutations in the malaria vector *Anopheles gambiae* from Cameroon. *American Journal of Tropical Medicine and Hygiene* **74**, 795–797.

Etang J, Manga L, Toto JC *et al.* (2007) Spectrum of metabolic-based resistance to DDT and pyrethroids in *Anopheles gambiae* s.l. populations from Cameroon. *Journal of Vector Ecology* **32**, 123–133.

Fanello C, Santolamazza F & della Torre A (2002) Simultaneous identification of species and molecular forms of the *Anopheles gambiae* complex by PCR-RFLP. *Medical and Veterinary Entomology* **16**, 461–464.

Fanello C, Petrarca V, della Torre A *et al.* (2003) The pyrethroid *knock-down* resistance gene in the *Anopheles gambiae* complex in Mali and further indication of incipient speciation within *An. gambiae s.s. Insect Molecular Biology* **12**, 241–245.

Georghiou GP, Breeland G & Ariaratnam V (1973) Seasonal escalation of organophosphorus and carbamate resistance in *Anopheles albimanus* by agricultural sprays. *Environmental Entomology* **2**, 369–374.

Giner M, Vassal C & Damiand G *et al.* (1999) *WINDL*, version 2.0. CIRAD (Centre de coopération Internationale en Recherche Agronomique pour le Développement, Montpellier, France.

Guillet P, N'Guessan R, Darriet F *et al.* (2001) Combined pyrethroid and carbamate 'two-in-one' treated mosquito nets: field efficacy against pyrethroid-resistant *Anopheles gambiae* and *Culex quinquefasciatus*. *Medical and Veterinary Entomology* **15**, 105–112.

Guyatt HL, Corlett SK, Robinson TP *et al.* (2002) Malaria prevention in highland Kenya: indoor residual house-spraying vs. insecticide-treated bednets. *Tropical Medicine and International Health* **7**, 298–303.

Hargreaves K, Koekemoer LL, Brooke B *et al.* (2000) *Anopheles funestus* resistant to pyrethroid insecticides

in South Africa. *Medical and Veterinary Entomology* **14**, 181–189.

Hayes JD & Pulford DJ (1995) The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Critical Review of Biochemistry and Molecular Biology* **30**, 445–600.

Hemingway J & Karunaratne SHP (1998) Mosquito carboxylesterases: a review of molecular biology and biochemistry of a major resistance mechanism. *Medical and Veterinary Entomology* **12**, 1–12.

Hemingway J & Ranson H (2000) Insecticide resistance in insect vectors of human disease. *Annual Review of Entomology* **45**, 371–391.

Hemingway J, Hawkes NJ, McCarroll L *et al.* (2004) The molecular basis of insecticide resistance in mosquitoes. *Insect Biochemistry and Molecular Biology* **34**, 653–665.

Holt RA, Subramanian GM, Halpern A *et al.* (2002) The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science* **298**, 129–149.

Huang H, Stok JE, Stoutamire DW *et al.* (2005) Development of optically pure pyrethroid-like fluorescent substrates for carboxylesterases. *Chemistry, Resistance and Toxicology* **18**, 516–527.

Kolaczinski JH, Fanello C, Hervé JP *et al.* (2000) Experimental and molecular genetic analysis of the impact of pyrethroid and non pyrethroid insecticide impregnated bednets for mosquito control in an area of pyrethroid resistance. *Bulletin of Entomological Research* **90**, 125–132.

Li X, Mary A, Schuler MA *et al.* (2007) Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annual Review of Entomology* **52**, 231–253.

Lines JD (1988) Do agricultural insecticides select for insecticide resistance in mosquitoes? A look at the evidence. *Parasitology Today* **7**, S17–S20.

Lynd A, Ranson H, McCall PJ *et al.* (2005) A simplified method for pyrethroid knockdown resistance (kdr) detection in *Anopheles gambiae*. *Malaria Journal* **4**, 16.

Martin T, Chandre F, Ochou OG *et al.* (2002) Pyrethroid resistance mechanisms in the cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae) from West Africa. *Pesticide Biochemistry and Physiology* **74**, 17–26.

Martinez-Torres D, Chandre F, Williamson MS *et al.* (1998) Molecular characterisation of pyretroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae s.s. Insect Molecular Biology* **7**, 179–184.

McAbee RD, Kang K-D, Stanich M *et al.* (2003) Pyrethroid tolerance in *Culex pipiens var molestus* from Marin County, California. *Pesticide Management Science* **60**, 359–368.

Müller P, Donnelly MJ & Ranson H (2007) Transcription profiling of a recently colonised pyrethroid resistant *Anopheles gambiae* strain from Ghana. *BMC Genomics* **8**, 36.

N'Guessan R, Corbel V & Akogbéto M *et al.* (2007) Reduced efficacy of insecticide treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin. *Emerging Infectious Diseases* **13** 199–206.

Najera JA & Zaim M (2001) *Malaria Vector Control – Insecticides for Indoor Residual Spraying. WHO/CDS/WHO-PES/2001.3*.

Pedra JHF, McIntyre LM, Scharf ME *et al.* (2004) Genome-wide transcription profile of field- and laboratory-selected dichlorodiphenyltrichloroethane (DDT)- resistant *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 7034–7039.

Penilla RP, Rodriguez RD, Hemingway J *et al.* (1998) Resistance management strategies in malaria vector mosquito control. Baseline data for a large-scale field trial against *Anopheles albimanus* in Mexico. *Medical and Veterinary Entomology* **12**, 217–233.

Pinto J, Lynd A, Elissa N *et al.* (2006) Co-occurrence of East and West African kdr mutations suggests high levels of resistance to pyrethroid insecticides in *Anopheles gambiae* from Libreville, Gabon. *Medical and Veterinary Entomology* **20**, 27–32.

Ranson H, Jensen B, Vulule JM *et al.* (2000a) Identification of a point mutation in the voltage-gated sodium channel gene of *Anopheles gambiae* associated with resistance to DDT and pyrethroids. *Insect Molecular Biology* **9**, 491–497.

Ranson H, Jensen B, Wang X *et al.* (2000b) Genetic mapping of two loci affecting DDT resistance in the malaria vector *Anopheles gambiae*. *Insect Molecular Biology* **9**, 499–507.

Stump AD, Atieli FK, Vulule JM *et al.* (2004) Dynamics of the pyrethroids knockdown resistance in Western Kenyan populations of *Anopheles gambiae* in response to insecticide-treated bed net trials. *American Journal of Tropical Medicine and Hygiene* **70**, 591–596.

Tripet F, Wright J, Cornel A *et al.* (2007) Longitudinal survey of knockdown resistance to pyrethroid (*KDR*) in Mali, West Africa, and evidence of its emergence in the Bamako form of *Anopheles gambiae s.s. American Journal of Tropical Medicine and Hygiene* **76**, 81–87.

Vontas JG, Small GJ & Hemingway J (2001) Glutathione-S-transferase as antioxidant defense agents confer pyrethroid resistance in *Nilaparvata lugens*. *Biochemistry Journal* **357**, 65–72.

Vontas J, Blass C, Koutsos AC *et al.* (2005) Gene expression in insecticide resistant and susceptible *Anopheles gambiae* strains constitutively or after insecticide exposure. *Insect Molecular Biology* **14**, 509–521.

WHO (1970) *Résistance aux Insecticides et Lutte Antivectorielle. 17*e Rapport Technique du Comité OMS d'experts des Insecticides. Series No 443. WHO, Geneva 306 pp.

WHO (1998) *Tests Procedures for Insecticide Resistance Monitoring in Malaria Vectors, Bioefficacy and Persistence of Insecticides on Treated Surfaces. Report of the WHO Informal Consultation, WHO/MAL/98*. Geneva, 43 pp.

WHO (2002) *Scaling-up Insecticide Treated Netting Programmes in Africa: A Strategic Framework for Coordinated National Action. WHO/CDS/RBM/2002.43*.

WHO (2004) *GLOBAL STRATEGIC FRAMEWORK for Integrated Vector Management. WHO/CDS/CPE/PVC/2004.10*.

WHO (2006) *Pesticides and Their Application for the Control of Vectors and Pests of Public Health Importance. WHO/CDS/NTD/WHOPES/GCDPP/2006.1.*

Wondji C, Simard F, Petrarca V *et al.* (2005) Species and populations of the *Anopheles gambiae* complex in Cameroon with special emphasis on chromosomal and molecular forms of *Anopheles gambiae s.s. Journal of Medical Entomology* **42**, 998–1005.

Zaim M & Guillet P (2002) Alternative insecticides: an urgent need. *Trends in Parasitology* **18**, 161–163.

Zaim M, Atio A & Nakashima N (2000) Safety of pyrethroid-treated nets. *Medical and Veterinary Entomology* **14**, 1–5.

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