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Ecological variation and resistance levels to propoxur and chlorpyrifos in *Anopheles stephensi* (Diptera: Culicidae), a malaria mosquito from India

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Abstract. A total of 39 strains of *Anopheles stephensi*, an important urban malaria vector, were collected from various parts of India and maintained in the insectary for this study. Based on the egg-float ridge number, 19 strains were classified into ecological variants and 32 strains were exposed to chlorpyrifos and propoxur to investigate their resistance status. Filter paper containing freshly laid eggs was taken, the ridge numbers on the floats were counted under the microscope, and strains were classified into ecological variants. Of the 19 strains, 18 were of 'type form', with ridge numbers ranging from 15 to 21. The Papareddipalya (PRP) strain belonged to the 'intermediate form', with 14 to 17 ridge numbers. Larval bioassays were carried out according to the procedure of the WHO. For chlorpyrifos, the lowest LC_{50} value was 0.00107 mg/l (Padmanabhanagar strain) and the highest value was 0.0403 mg/l (GOA-A strain). Furthermore, the lowest LC_{90} value was 0.00368 mg/l (Delhi strain) and the highest was 0.1746 mg/l (GOA-A strain). For propoxur, the lowest LC_{50} value was 0.00029 mg/l (Goraguntepalya strain) and the highest value was 0.0037 mg/l (JP Nagar strain). Moreover, the lowest LC_{90} value was 0.00094 mg/l (Goraguntepalya strain) and the highest value was 0.0115 mg/l (JP Nagar strain). The tolerance values ranged from 1.26 to 37.68 for chlorpyrifos and from 1.34 to 12.77 for propoxur. All the type forms were from urban and semi-urban locations, and the intermediate strain was from a semi-urban location. The bioassay results indicated that the strains of *An. stephensi* were more susceptible to propoxur than to chlorpyrifos.

Key words: *Anopheles stephensi*, chlorpyrifos, egg-float ridge number, larval bioassay, type form, intermediate form, propoxur

Introduction

Mosquito-borne diseases take a heavy toll on human lives. Due to its blood-sucking behaviour, a female mosquito is able to transmit pathogens and parasites that cause serious diseases including malaria, filariasis, dengue haemorrhagic fever, Japanese encephalitis, yellow fever and chikungunya. These emerging and resurging diseases result in high burden of disease that reflects inadequate implementation and/or impact from current control measures (WHO, 2009).

According to the latest estimates, there were about 207 million cases of malaria in 2012 and an estimated 0.62 million deaths (WHO, 2014).

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In India, 0.2 million deaths have been reported to occur annually due to malaria (Dhingra *et al.*, 2010).

Medically important species of mosquito belong to three genera: *Anopheles*; *Aedes*; *Culex*. About 455 named species and 40 unnamed members of complexes recognized as distinct morphological or genetic species of *Anopheles* have been identified (Harbach, 2007). In India, about 58 species of *Anopheles* are prevalent, of which six are primary vectors of malaria and four are secondary vectors (Nagpal and Sharma, 1995). The six primary vectors are distributed as follows: *Anopheles culicifacies* in rural areas, *An. stephensi* in urban settings, *An. fluviatilis* in plains and foothills, *An. minimus* in foothills of the northeast, *An. dirus* in jungles of northeastern states and *An. sundaicus* in the islands (Andaman and Nicobar) (Dev and Sharma, 2013). *Anopheles stephensi* Liston (Diptera: Culicidae), the primary urban vector, has been reported to account for about 15% of the incidents of malaria in India (Shetty, 2002*a*).

Anopheles stephensi was classified as two geographical races based on the number of ridges on the egg float; urban *An. stephensi* was regarded as the type form and the rural variety as *mysorensis*. Later, several examinations revealed the presence of three variants: type form; *mysorensis*; intermediate (Sweet and Rao, 1937; Rao *et al.*, 1938). The numbers of ridges for the variants are 14–22 (type form), 9–15 (*mysorensis*) and 12–17 (intermediate). The type and intermediate forms have been found in urban and semi-urban areas and reported to be vectors; the *mysorensis* form has been found to be predominant in rural areas and reported as a non-vector (Subbarao *et al.*, 1987; Shetty *et al.*, 1999). The type form has been found to be exclusively domestic in all seasons, whereas *mysorensis* occupies the outdoor niche during monsoon and post-monsoon seasons with a spillover into domestic sites during summer periods of ecological stress (Nagpal *et al.*, 2003).

Due to urbanization, there is a surge in construction activity. This creates ideal conditions for mosquito breeding, and the migration from endemic areas accounts for the high number of incidents of mosquito-borne diseases; therefore, either the parasites or the vectors should be controlled. The strategy adopted to curtail mosquito-borne disease transmission in urban settings is through anti-larval operations (Tiwari *et al.*, 2010). There is a widespread use of insecticides because they are effective, convenient to apply and economical.

Indiscriminate application of insecticides leads to the development of resistance. Susceptibility studies act as a resource in resistance surveillance, which provides baseline data for programme planning, insecticide dose selection, detection of resistant individuals at an early stage, and continuous monitoring of the effect of insecticides on resistance (WHO, 1981; National Research Council, 1986). The determination of general resistance spectra is the first stage in the investigation of any insecticide-resistant population (Hemingway, 1981). For this purpose, the indigenous mosquito species should be collected and their minimum effective dosages such as LC_{50} and LC_{90} values for different insecticides should be

Table 1. Strains of *Anopheles stephensi* collected from different geographical regions of India

Serial no.	Locality	Strain code	Collected as
1	Aurangabad	AGB[1]	Larvae
2	Bannerghatta Road[2]	BGR[1]	Larvae
3	Basaveshwaranagar[2]	BSN[1,3]	Larvae
4	BTM Layout[2]	BTM[3]	Larvae
5	Cambridge Layout[2]	CLO[1]	Larvae
6	Chamarajpet[2]	CRP[1,3]	Larvae
7	Chennai	CHN[1]	Larvae
8	Delhi	DEL[1]	Larvae
9	Dollar's Colony[2]	DLN[1,3]	Larvae
10	Gandhinagar[2]	GDN[1,3]	Larvae
11	Gandhinagar-Mysore	MYS[1]	Larvae
12	Goa-A	GOA-A[1]	Larvae
13	Goa-B	GOA-B[1]	Larvae
14	Goraguntepalya[2]	GGP[1]	Larvae
15	Hebbal[2]	HBB[1,3]	Larvae
16	JP Nagar[2]	JPN[1,3]	Larvae
17	Jnanabharathi Campus[2]	JBC[1,3]	Larvae
18	Jodhpur	JDP[3]	Larvae
19	Kengeri[2]	KGR[1,3]	Larvae/adults
20	Kolar	KLR[3]	Larvae/adults
21	Mahalakshmipuram[2]	MLP[1]	Larvae
22	Mangalore I	K1[3]	Larvae
23	Mangalore II	K2[3]	Larvae
24	Mangalore III	K3[3]	Larvae
25	Mangalore-A	MGL-A[1]	Larvae
26	Mangalore-B	MGL-B[1]	Larvae
27	Murgeshpalya[2]	MGP[3]	Larvae
28	Nelamangala	NMG[1,3]	Larvae
29	Padmanabhanagar[2]	PBN[1]	Larvae
30	Papareddipalya[2]	PRP[1,3]	Larvae/adults
31	Pondicherry	PDC[1]	Larvae
32	Pune-A	PUN-A[1]	Larvae
33	Pune-B	PUN-B[1]	Larvae
34	Punjab	PNJ[1]	Larvae
35	Shimoga	SMG[1]	Larvae
36	Subashnagar[2]	SBN[1]	Larvae
37	Tumkur	TMK[1,3]	Larvae
38	West of Chord Road[2]	WCR[1]	Larvae
39	Yeshwanthpur[2]	YSP[1,3]	Larvae

[1] Strains used for larval bioassay.
[2] Strains from Bangalore.
[3] Strains used for egg-float ridge number studies.

evaluated. Based on the results obtained in the laboratory, an effective optimum dosage for field applications can be determined. The status of insecticide susceptibility/resistance has to be reviewed periodically to either continue or discontinue specific insecticides. Vector control operations require monitoring insecticide susceptibility to determine dosage, establish baseline levels for future resistance work, and evaluate the effects of insecticides on disease incidence and vector behaviour.

Therefore, the aim of the present work was to study the ecological variations in *An. stephensi* based on the egg-float ridge numbers in different populations, and also their resistance status under laboratory conditions to two insecticides: chlorpyrifos (an organophosphate insecticide) and propoxur (a carbamate insecticide).

Fig. 1. Map of Bangalore, Karnataka, showing the mosquito collection sites. 1, Bannerghatta Road; 2, Basaveshwaranagar; 3, BTM Layout; 4, Cambridge Layout; 5, Chamarajpet; 6, Dollar's Colony; 7, Gandhinagar; 8, Goraguntepalya; 9, Hebbal; 10, JP Nagar; 11, Jnanabharathi Campus; 12, Kengeri; 13, Mahalakshmipuram; 14, Murgeshpalya; 15, Padmanabhanagar; 16, Papareddipalya; 17, Subashnagar; 18, West of Chord Road; 19, Yeshwanthpur.

Materials and methods

Anopheles stephensi – *strains and maintenance*

A total of 39 strains of *An. stephensi* from various parts of India (Table 1) were used in this study, of which 19 were from Bangalore (Fig. 1) and 20 from other parts of the country (Fig. 2). Colonies were maintained in the insectary following the procedure of Shetty (1983), in cages with iron frames covered with cotton mosquito net. Adults were fed with 10% sucrose solution on soaked sterilized cotton, and females were provided with blood meal on restrained mice 5 days after their emergence. Water-filled plastic cups lined with filter paper were placed inside the cages for oviposition. Gravid females laid eggs 48 h after taking the blood meal. The eggs were kept for 72 h to ensure complete hatching. Larvae were reared in white enamel pans containing filtered tap water and fed with powdered yeast tablets on a regular schedule throughout the larval period. To avoid scum formation, water in the pans was changed every day. Pupation began 8–10 days after hatching. Pupae were transferred into wide-mouthed bottles and emerging adults were released into their respective cages. These stocks were maintained at a temperature of 25 ± 1 °C with relative humidity of 75 ± 5% and 10 h of photoperiod throughout the course of investigations.

Egg-float ridge number

For each strain, the fresh (unhatched) eggs laid by the blood-fed females, along with the filter paper

Fig. 2. Map of India showing the mosquito collection sites. 1, Aurangabad; 2, Chennai; 3, Delhi; 4, Gandhinagar-Mysore; 5, Goa-A; 6, Goa-B; 7, Jodhpur; 8, Kolar; 9, Mangalore I; 10, Mangalore II; 11, Mangalore III; 12, Mangalore A; 13, Mangalore B; 14, Nelamangala; 15, Pondicherry; 16, Pune-A; 17, Pune-B; 18, Punjab; 19, Shimoga; 20, Tumkur.

provided for oviposition, were placed under the microscope for counting the egg-float ridge numbers (10× magnification, LABO, Bioplan XL, Jupiter Scientific Company, Salem, Tamil Nadu, India). Based on the number of ridges on the egg floats, the strains were grouped into type (14–22 ridges), *mysorensis* (9–15) and intermediate (12–17). The percentage distribution of ridges in each strain was calculated according to the procedure of Shetty *et al.* (1999).

Insecticides

Two insecticides were used in the present study: chlorpyrifos (an organophosphate insecticide) and propoxur (a carbamate insecticide).

Chlorpyrifos (21.5% E.C.) is one of the most commonly used insecticides in agriculture, horticulture and mosquito control. It is non-systemic and kills by direct contact or ingestion. The IUPAC nomenclature is *O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridylphosphorothioate with the molecular formula of $C_9H_{11}C_{l3}NO_3P$. It acts on the nervous system of insects by inhibiting acetylcholinesterase.

Propoxur (Baygon – 2% E.C.) is a crystalline derivative of carbamic acid. It is a non-systemic, contact and stomach poison and used against mosquitoes in outdoor areas. Propoxur is one of the chemicals that has, to a large extent, replaced DDT in mosquito control (McEwen and Stephenson, 1979). It has residual poisonous or toxic activity when it is in direct contact with the target pest (Hartley and Kidd, 1983). The IUPAC nomenclature is 2-isopropoxyphenyl methylcarbamate with the molecular formula of $C_{11}H_{15}NO_3$. It has been approved by and registered with the Central Insecticide Board and Registration Committee, the Ministry of Agriculture, Government of India, the WHO, and Environmental Protection Agency, USA, for use against household pests including mosquito. As with other carbamates, propoxur blocks the production and action of cholinesterase, paralyzing the nervous system of insects and causing a rapid 'knockdown' effect.

Larval bioassays

Susceptibility tests were carried out according to the procedure of WHO (2005). Different concentrations (mg/l) of chlorpyrifos were prepared in denatured alcohol (98 ml absolute alcohol + 2 ml ethyl methyl ketone) and propoxur in water. A total of 25 late third-instar larvae were transferred into glass bottles, each containing the test concentration (249 ml of dechlorinated tap water + 1 mg/l stock concentration), with four replicates. Mortality was assessed after 24 h. Mortality data from bioassays were corrected by natural control mortality using Abbot's formula (Abbott, 1925); LC_{50} and LC_{90} were calculated by log-dose probit analysis (Finney, 1971). We set up a control by adding 1 ml of denatured alcohol/water to 249 ml water. If more than 10% of the larvae pupated during the course of the experiment, the test was discarded.

Table 2. Percentage distribution of egg-float ridge numbers for the different strains of *Anopheles stephensi*

			No. of ridges on egg float										
Serial no.	Strain	No. of eggs	14	15	16	17	18	19	20	21	Range	Mean ± SD	Variety
1	BTM	100				69		31			17–19	17.62 ± 0.929	Type
2	BSN	100			4	24	72				16–18	17.68 ± 0.548	Type
3	CRP	100				4	32	64			17–19	18.6 ± 0.568	Type
4	DLN	100			19	81					16–17	16.81 ± 0.394	Type
5	GDN	169			17.8	59.2	19.5	3.5			16–19	17.08 ± 0.714	Type
6	HBB	157		14.0	58.6	17.8	9.6				15–18	16.22 ± 0.807	Type
7	JPN	100						12	9	79	19–21	20.67 ± 0.682	Type
8	JBC	159			22.0	63.5	14.5				16–18	16.92 ± 0.601	Type
9	JDP	163			18.4	38.7	24.5	18.4			16–19	17.42 ± 0.993	Type
10	K1	104		26	32.7	23	12.5	5.8			15–19	16.39 ± 1.169	Type
11	K2	124		15.3	22.5	21	30.6	10.5			15–19	16.98 ± 1.255	Type
12	K3	135			12.6	60.0	21.5	5.9			16–19	17.20 ± 0.733	Type
13	KGR	147			25.2	49.7	15.6	9.5			16–19	17.09 ± 0.886	Type
14	KLR	197			18.8	61.9	19.3				16–18	17.0 ± 0.618	Type
15	MGP	210				12.9	24.3	51.4	11.4		17–20	18.60 ± 0.842	Type
16	NMG	100				7		17	3	73	17–21	20.35 ± 1.192	Type
17	PRP	172	19.8	65.7	10.5	4.0					14–17	14.98 ± 0.683	Intermediate
18	TMK	100			77	23					16–17	16.23 ± 0.422	Type
19	YSP	100			12	83				5	16–21	17.08 ± 0.960	Type

The tolerance/resistance ratio was calculated by dividing the LC_{50}/LC_{90} value of a strain by the LC_{50}/LC_{90} value of the least resistant strain for each insecticide (Boike *et al.*, 1989).

Results

The various strains of *An. stephensi* used in this study along with the strain codes are presented in Table 1. The ridges on the egg floats of the 19 strains were counted and the percentage distribution calculated for each strain. Based on the number of ridges, each strain was classified as type form, intermediate form or *mysorensis*. Of the 19 strains studied, 18 (BTM, BSN, CRP, DLN, GDN, HBB, JPN, JBC, JDP, K1, K2, K3, KGR, KLR, MGP, NMG, TMK and YSP) were classified as type form. The PRP strain was classified as intermediate form (Table 2).

Larval susceptibility status to chlorpyrifos is presented (Table 3 and Fig. 3). Among the strains, GOA-A showed the highest LC_{50} value (4×10^{-2} mg/l) followed by GGP (3.474×10^{-2} mg/l), SBN (2.871×10^{-2} mg/l), GDN (2.644×10^{-2} mg/l) and BSN (2.479×10^{-2} mg/l); the lowest LC_{50} was shown by PBN (1.07×10^{-3} mg/l) followed by PUN-A (1.35×10^{-3} mg/l), DEL (1.42×10^{-3} mg/l), JPN (1.5×10^{-3} mg/l) and DLN (1.68×10^{-3} mg/l). The GOA-A strain showed the highest LC_{90} value (1.7466×10^{-1} mg/l), followed by GGP (1.3896×10^{-1} mg/l), SBN (1.013×10^{-1} mg/l), GDN (1.003×10^{-1} mg/l) and PNJ (9.638×10^{-2} mg/l); the lowest LC_{90} value was shown by DEL (3.68×10^{-3} mg/l), followed by PBN

Table 3. LC_{50}, LC_{90}, regression equation, coefficient of correlation, χ^2 and tolerance/resistance ratio (RR) values of chlorpyrifos for the different strains of *Anopheles stephensi*

Strains	LC_{50} (mg/l)	LC_{90} (mg/l)	Regression equation	r	$\chi^{2\,1}$	RR	
						$LC_{50}{}^2$	$LC_{90}{}^3$
AGB	1.98×10^{-3}	1.022×10^{-2}	$y = 1.7974x + 0.871$	0.9566	2.7481 (df = 5)	1.8527	2.7762
BGR	1.096×10^{-2}	5.834×10^{-2}	$y = 1.7633x - 0.3605$	0.9907	0.9360 (df = 5)	10.2475	15.8545
BSN	2.479×10^{-2}	8.937×10^{-2}	$y = 2.2983x - 2.801$	0.9956	2.2677 (df = 5)	23.1641	24.2858
CHN	4.3×10^{-3}	1.787×10^{-2}	$y = 2.0705x - 0.4535$	0.9438	2.1581 (df = 5)	4.0227	4.8557
CLO	2.54×10^{-3}	9.7×10^{-3}	$y = 2.1982x - 0.2851$	0.9970	1.2346 (df = 5)	2.3709	2.6348
CRP	2.33×10^{-3}	1.805×10^{-2}	$y = 1.4405x + 1.5891$	0.9348	2.7854 (df = 6)	2.1803	4.9040
DEL	1.42×10^{-3}	3.68×10^{-3}	$y = 3.0845x - 1.636$	0.9706	0.4982 (df = 5)	1.3244	1.0013
DLN	1.68×10^{-3}	6.91×10^{-3}	$y = 2.0816x + 0.369$	0.9577	2.3403 (df = 5)	1.5679	1.8782
GDN	2.644×10^{-2}	1.003×10^{-1}	$y = 2.2107x - 2.5656$	0.9878	1.3474 (df = 5)	24.7125	27.2554
GGP	3.474×10^{-2}	1.3896×10^{-1}	$y = 2.126x - 2.5277$	0.9793	0.2048 (df = 5)	32.4651	37.7618
GOA-A	4.033×10^{-2}	1.7466×10^{-1}	$y = 2.0109x - 2.2506$	0.9507	0.6520 (df = 6)	37.6891	47.4627
GOA-B	1.693×10^{-2}	5.789×10^{-2}	$y = 2.397x - 2.7389$	0.9425	0.2275 (df = 5)	15.8204	15.7308
HBB	9.81×10^{-3}	3.085×10^{-2}	$y = 2.5723x - 2.6955$	0.9713	0.4648 (df = 5)	9.1689	8.3840
JBC	3.62×10^{-3}	1.057×10^{-2}	$y = 2.7509x - 2.0387$	0.9338	1.3622 (df = 5)	3.3831	2.8718
JPN	1.5×10^{-3}	1.151×10^{-2}	$y = 1.4484x + 1.8466$	0.9773	0.9218 (df = 5)	1.4055	3.1265
KGR	1.345×10^{-2}	4.111×10^{-2}	$y = 2.6378x - 3.2527$	0.9816	0.7850 (df = 5)	12.5666	11.1700
MGL-A	3.72×10^{-3}	2.155×10^{-2}	$y = 1.6788x + 0.6838$	0.9771	0.7109 (df = 6)	3.4803	5.8567
MGL-B	1.76×10^{-3}	7.96×10^{-3}	$y = 1.9564x + 0.6045$	0.9833	4.2904 (df = 6)	1.6494	2.1635
MLP	2.55×10^{-3}	9.14×10^{-3}	$y = 2.307x - 0.5511$	0.9935	0.4956 (df = 5)	2.3813	2.4840
MYS	2.94×10^{-3}	9.36×10^{-3}	$y = 2.5466x - 1.2873$	0.9640	1.8356 (df = 5)	2.7512	2.5448
NMG	6.16×10^{-3}	2.437×10^{-2}	$y = 2.142x - 0.9747$	0.9819	0.9831 (df = 6)	5.7533	6.6230
PBN	1.07×10^{-3}	4.38×10^{-3}	$y = 2.0891x + 0.7608$	0.9585	2.4577 (df = 5)	0.9996	1.1914
PDC	3.44×10^{-3}	1.085×10^{-2}	$y = 2.564x - 1.5027$	0.9699	1.6440 (df = 5)	3.2123	2.9482
PNJ	7.88×10^{-3}	9.638×10^{-2}	$y = 1.7711x + 1.5904$	0.9818	1.1768 (df = 5)	7.3657	26.1910
PRP	2.165×10^{-2}	7.019×10^{-2}	$y = 2.5058x - 3.3581$	0.9572	1.4460 (df = 5)	20.2356	19.0745
PUN-A	1.35×10^{-3}	7.92×10^{-3}	$y = 1.6689x + 1.4423$	0.9757	1.6949 (df = 5)	1.2658	2.1520
PUN-B	2.82×10^{-3}	8.44×10^{-3}	$y = 2.6914x - 1.5963$	0.9728	2.6132 (df = 5)	2.6395	2.2943
SBN	2.871×10^{-2}	1.013×10^{-1}	$y = 2.3377x - 3.0838$	0.9650	0.0375 (df = 6)	26.8297	27.5266
SMG	3.26×10^{-3}	1.216×10^{-2}	$y = 2.2373x - 0.6224$	0.9685	3.1039 (df = 5)	3.0452	3.3056
TMK	1.86×10^{-3}	7.8×10^{-3}	$y = 2.0519x - 0.3454$	0.9689	0.9827 (df = 6)	1.7339	2.1201
WCR	1.619×10^{-2}	6.552×10^{-2}	$y = 2.1079x - 1.7646$	0.9529	3.6451 (df = 5)	15.1292	17.8054
YSP	4.35×10^{-3}	1.662×10^{-2}	$y = 2.1996x - 0.8043$	0.9819	0.6853 (df = 6)	4.0683	4.5170

[1] Statistically non-significant ($P < 0.05$).
[2] Tolerance $RR_{50} = LC_{50}$ of a strain/LC_{50} of the PBN strain.
[3] Tolerance $RR_{90} = LC_{90}$ of a strain/LC_{90} of the DEL strain.

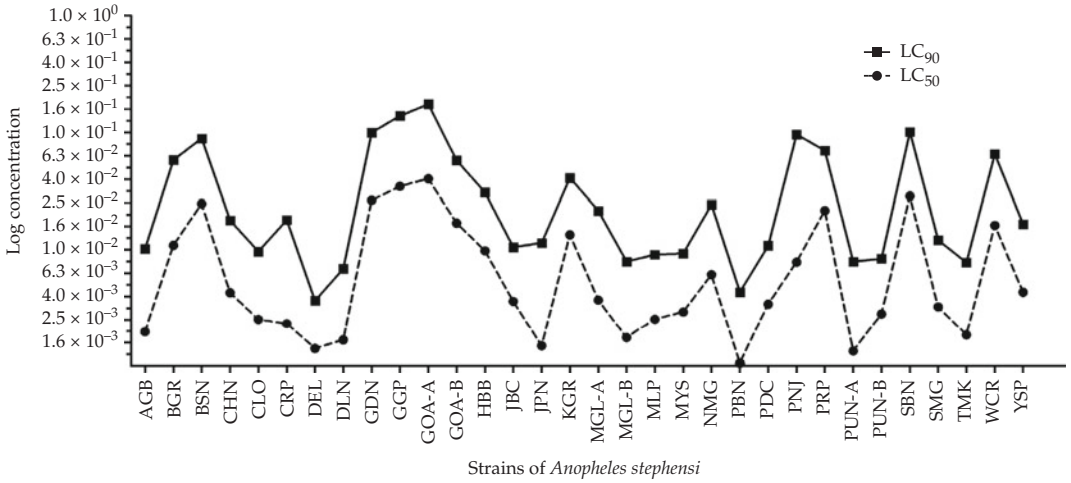

Fig. 3. Susceptibility status of different strains of *Anopheles stephensi* to chlorpyrifos.

(4.38×10^{-3} mg/l), DLN (6.91×10^{-3} mg/l), TMK (7.80×10^{-3} mg/l) and PUN-A (7.92×10^{-3} mg/l). χ^2 values were found to be non-significant at $P < 0.05$.

Larval susceptibility status to propoxur is presented (Table 4 and Fig. 4). Among the strains, JPN showed the highest LC_{50} value (3.7×10^{-3} mg/l), followed by YSP (2.16×10^{-3} mg/l), JBC (1.88×10^{-3} mg/l), AGB (1.46×10^{-3} mg/l) and PRP (1.04×10^{-3} mg/l); GGP showed the lowest LC_{50} value (2.9×10^{-4} mg/l), followed by PDC (3.9×10^{-4} mg/l), MGL-B and PUN-A (4×10^{-4} mg/l) and CRP (4.2×10^{-4} mg/l). JPN showed the highest LC_{90} value (1.159×10^{-2} mg/l), followed by YSP (7.64×10^{-3} mg/l), JBC (6.16×10^{-3} mg/l), AGB (5.62×10^{-3} mg/l) and WCR (4.75×10^{-3} mg/l); GGP showed the lowest LC_{90} value (9.4×10^{-4} mg/l), followed by PDC (1.07×10^{-3} mg/l), CRP (1.15×10^{-3} mg/l), MGL-B (1.17×10^{-3} mg/l) and PUN-A (1.34×10^{-3} mg/l). χ^2 values were found to be non-significant at $P < 0.05$.

For chlorpyrifos, the LC_{50} values ranged from 1.07×10^{-3} to 4×10^{-2} mg/l and the LC_{90} values ranged from 3.68×10^{-3} to 1.7466×10^{-1} mg/l. Its mean ± SD LC_{50} value was $9.57 \times 10^{-3} \pm 0.0109$ mg/l and its LC_{90} value was 3.91×10^{-2} 0.044 mg/l. For propoxur, the LC_{50} values ranged from 2.9×10^{-4} to 3.7×10^{-3} mg/l and the LC_{90} values ranged from 9.4×10^{-4} to 1.159×10^{-2} mg/l. Its mean ± SD LC_{50} value was $8.2 \times 10^{-4} \pm 0.00066$ mg/l and its LC_{90} value was $2.8 \times 10^{-3} \pm 0.0022$ mg/l (Table 5).

For chlorpyrifos, the lowest LC_{50} tolerance values were 1.26 (PUN-A), 1.32 (DEL), 1.40 (JPN), 1.56 (DLN) and 1.64 (MGL-B); the highest values were 37.68 (GOA-A), 32.46 (GGP), 26.82 (SBN), 24.71 (GDN) and 23.16 (BSN) (Table 3). The lowest LC_{90} tolerance values were 1.19 (PBN), 1.87 (DLN), 2.12 (TMK), 2.15 (PUN-A) and 2.16 (MGL-B); the highest values were 47.46 (GOA-A), 37.76 (GGP), 27.52 (SBN), 27.25 (GDN) and 26.19 (PNJ) (Table 3). The LC_{50} tolerance values ranged from 1.26 to 37.68, with a mean ± SD of 8.9526 ± 10.198. The LC_{90} tolerance values ranged from 1.19 to 47.46, with a mean ± SD of 10.6484 ± 11.9642 (Table 6).

For propoxur, the lowest LC_{50} tolerance values were 1.34 (PDC), 1.37 (MGL-B), 1.39 (CRP), 1.44 (CRP) and 1.48 (MGL-A); the highest values were 12.77 (JPN), 7.45 (YSP), 6.47 (JBC), 5.02 (AGB) and 3.57 (PRP). The lowest LC_{90} tolerance values were 1.13 (PDC), 1.22 (CRP), 1.24 (MGL-B), 1.42 (PUN-A) and 1.64 (PNJ); the highest values were 12.32 (JPN), 8.12 (YSP), 6.55 (JBC), 5.98 (AGB) and 5.05 (WCR) (Table 4). The LC_{50} tolerance values ranged from 1.34 to 12.77, with a mean ± SD of 2.8361 ± 2.3066. The LC_{90} tolerance values ranged from 1.13 to 12.32, with a mean ± SD of 3.0720 ± 2.3443 (Table 6).

From the present study, we found that the strains BGR, BSN, GDN, GGP, GOA-A, GOA-B, KGR, PRP, SBN and WCR were resistant (>10-fold tolerant to chlorpyrifos), while all were susceptible to propoxur except JPN, with a tolerance level of 12.77. The results indicate that the strains of *An. stephensi* were more susceptible to propoxur than to chlorpyrifos.

Discussion

The marked differences in the behaviour of *An. stephensi* had led to the existence of biological races being proposed. Two populations, type form and *mysorensis*, were reported on the basis of differences in egg length, width and number of ridges on the egg float (Sweet and Rao, 1937). Puri (1949) designated them as sub-species; however, Rutledge *et al.* (1970) found the two forms to be sympatric and interbreeding and thus considered them variants

Table 4. LC_{50}, LC_{90}, regression equation, coefficient of correlation, χ^2 and tolerance/resistance ratio (RR) values of propoxur for the different strains of *Anopheles stephensi*

Strains	LC_{50} (mg/l)	LC_{90} (mg/l)	Regression equation	r	$\chi^{2\,1}$	RR	
						$LC_{50}{}^2$	$LC_{90}{}^3$
AGB	1.46×10^{-3}	5.62×10^{-3}	$y = 2.1828x + 0.2775$	0.9807	0.7124 (df = 5)	5.0246	5.9810
BGR	4.8×10^{-4}	2.07×10^{-3}	$y = 2.0266x + 1.5862$	0.9992	0.0249 (df = 6)	1.6676	2.2028
BSN	8.3×10^{-4}	2.2×10^{-3}	$y = 3.0058x - 0.7625$	0.9752	1.1446 (df = 5)	2.8491	2.3435
CHN	5.8×10^{-4}	1.66×10^{-3}	$y = 2.7835x + 0.0974$	0.9987	0.0911 (df = 5)	1.9902	1.7704
CLO	6.1×10^{-4}	2.49×10^{-3}	$y = 2.1002x + 1.2488$	0.9808	1.5599 (df = 6)	2.1072	2.6453
CRP	4.2×10^{-4}	1.15×10^{-3}	$y = 2.9149x + 0.2734$	0.9822	0.7370 (df = 5)	1.4425	1.2234
DEL	6.3×10^{-4}	2.13×10^{-3}	$y = 2.4322x + 0.6189$	0.9829	1.6470 (df = 6)	2.1822	2.2619
DLN	7.9×10^{-4}	2.59×10^{-3}	$y = 2.4787x + 0.2973$	0.9855	0.6874 (df = 5)	2.7215	2.7572
GDN	6×10^{-4}	1.96×10^{-3}	$y = 2.478x + 0.5982$	0.9886	0.7769 (df = 6)	2.0606	2.0882
GGP	2.9×10^{-4}	9.4×10^{-4}	$y = 2.5466x + 1.2594$	0.9640	0.9827 (df = 5)	1.0000	0.9963
GOA-A	6.9×10^{-4}	2.79×10^{-3}	$y = 2.1011x + 1.1408$	0.9948	0.4369 (df = 6)	2.3681	2.9708
GOA-B	4.8×10^{-4}	2.8×10^{-3}	$y = 1.6627x + 2.21$	0.9963	0.1947 (df = 5)	1.6429	2.9831
HBB	5×10^{-4}	1.57×10^{-3}	$y = 2.5912x - 0.5877$	0.9783	1.8710 (df = 5)	1.7394	1.6737
JBC	1.88×10^{-3}	6.16×10^{-3}	$y = 2.4803x - 0.6391$	0.9642	3.0947 (df = 6)	6.4724	6.5535
JPN	3.7×10^{-3}	1.159×10^{-2}	$y = 2.5847x - 1.6394$	0.9877	0.8062 (df = 6)	12.7733	12.3274
KGR	7.7×10^{-4}	2.93×10^{-3}	$y = 2.205x + 0.8402$	0.9866	0.4434 (df = 5)	2.6552	3.1180
MGL-A	4.3×10^{-4}	1.6×10^{-3}	$y = 2.2542x + 1.3138$	0.9884	0.9007 (df = 5)	1.4890	1.6981
MGL-B	4×10^{-4}	1.17×10^{-3}	$y = 2.7456x + 0.6055$	0.9719	1.2456 (df = 5)	1.3747	1.2407
MLP	7.5×10^{-4}	2.99×10^{-3}	$y = 2.1217x + 1.027$	0.9886	0.9115 (df = 6)	2.5716	3.1818
MYS	7.5×10^{-4}	2.99×10^{-3}	$y = 2.1217x + 1.027$	0.9886	0.9115 (df = 6)	2.5716	3.1818
NMG	5.2×10^{-4}	1.70×10^{-3}	$y = 2.4768x + 0.7587$	0.9939	0.4846 (df = 6)	1.7783	1.8033
PBN	6.7×10^{-4}	2.08×10^{-3}	$y = 2.6146x + 0.2215$	0.9962	0.0920 (df = 5)	2.3185	2.2084
PDC	3.9×10^{-4}	1.07×10^{-3}	$y = 2.9166x + 0.3603$	0.9847	0.9211 (df = 5)	1.3440	1.1391
PNJ	4.4×10^{-4}	1.55×10^{-3}	$y = 2.3632x + 1.1056$	0.9693	0.9076 (df = 5)	1.5329	1.6462
PRP	1.04×10^{-3}	3.42×10^{-3}	$y = 2.4725x + 0.0158$	0.9763	1.3701 (df = 5)	3.5769	3.6339
PUN-A	4×10^{-4}	1.34×10^{-3}	$y = 2.4549x + 1.0575$	0.9756	3.1487 (df = 5)	1.3919	1.4265
PUN-B	7.3×10^{-4}	2.95×10^{-3}	$y = 2.1018x + 1.0889$	0.9898	0.6337 (df = 6)	2.5027	3.1381
SBN	9.4×10^{-4}	3.22×10^{-3}	$y = 2.3954x + 0.274$	0.9878	0.5063 (df = 6)	3.2397	3.4212
SMG	5.1×10^{-4}	1.75×10^{-3}	$y = 2.4017x + 0.8929$	0.9885	0.5481 (df = 5)	1.7689	1.8615
TMK	5×10^{-4}	1.55×10^{-3}	$y = 2.615x + 0.5533$	0.9642	1.7263 (df = 5)	1.7302	1.6473
WCR	9.9×10^{-4}	4.75×10^{-3}	$y = 1.8795x + 1.2494$	0.9877	0.2273 (df = 5)	3.4127	5.0521
YSP	2.16×10^{-3}	7.64×10^{-3}	$y = 2.3338x - 0.4489$	0.9878	0.6131 (df = 5)	7.4542	8.1297

[1] Statistically non-significant ($P < 0.05$).
[2] Tolerance $RR_{50} = LC_{50}$ of a strain/LC_{50} of the GGP strain.
[3] $RR_{90} = LC_{90}$ of a strain/LC_{90} of the GGP strain.

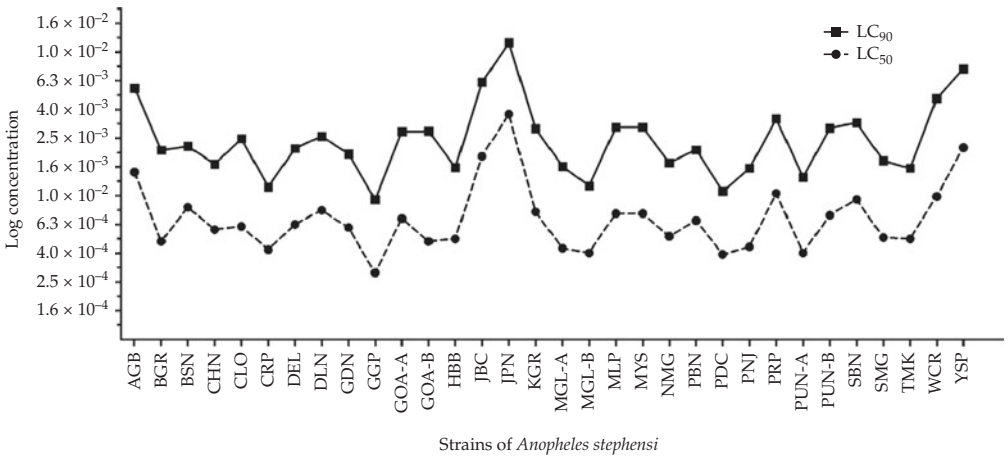

Fig. 4. Susceptibility status of different strains of *Anopheles stephensi* to propoxur.

Table 5. Range, mean ± SD and confidence limits (CL) of LC_{50} and LC_{90} for chlorpyrifos and propoxur

	LC_{50}			LC_{90}		
Insecticides	Range	Mean ± SD	95% CL	Range	Mean ± SD	95% CL
Chlorpyrifos ($n = 32$)	1.07×10^{-3} (PBN) to 4.03×10^{-2} (GOA-A)	9.57×10^{-3} ± 0.01	0.00 576–0.0133	3.68×10^{-3} (DEL) to 1.74×10^{-1} (GOA-A)	3.91×10^{-2} ± 0.04	0.02 379–0.05 457
Propoxur ($n = 32$)	2.9×10^{-4} (GGP) to 3.7×10^{-3} (JPN)	8.2×10^{-4} ± 0.0006	0.00 058–0.00 105	9.4×10^{-4} (GGP) to 1.159×10^{-2} (JPN)	2.8×10^{-3} ± 0.002	0.002117–0.00 365

and not sub-species. Classification of the vector into ecological variants has a propounding effect on disease transmission (Shetty *et al.*, 1999). The type and intermediate forms that are predominant in urban and semi-urban areas have been reported to be vectors, while *mysorensis*, reported to be predominant in rural areas, has been determined to be a non-vector (Sweet and Rao, 1937; Rao, 1984; Subbarao *et al.*, 1987; Shetty *et al.*, 1999). The result of the present study is in accordance with the earlier reports where type and intermediate forms have been found in urban and semi-urban areas. In the Jiroft district of Iran, *An. stephensi* was the *mysorensis* form, where the ridge number ranged from 10 to 14 (Mehravaran *et al.*, 2012).

Wide use of insecticide treatments is indispensable in almost all crop and public health programmes, especially those for vector control. The rational use of insecticides depends on a broad knowledge of the susceptibility and irritability levels of malaria vectors. This knowledge enables us to take all necessary precautions to prevent the occurrence of resistance, and to prepare in advance a plan for coping with it at the early stages of its development in the field (Vatandosst and Borhani, 2004).

To indicate a strain is resistant, a 10-fold increase in LC_{50} is necessary for mosquito larvae, while a 4-fold increase is sufficient for adult mosquito in comparison with the susceptible control (Brown and Pal, 1971). In the present study, a maximum 37.68-fold tolerance was observed in GOA-A compared with the least resistant strain (PBN) for chlorpyrifos and a 12.77-fold tolerance in JPN compared with the least resistant strain (GGP) for propoxur. Based on the results, it is clear that the tolerance values for chlorpyrifos and propoxur among many strains show more than a 10-fold tolerance, indicating resistance to these insecticides. The correlation coefficient (r) indicates that the observed values fall around the regression line. The r values are strong when placed between 0.8 and 1.0. All the r values obtained in the present study are >0.9, indicating close proximity to the regression line.

Insecticide susceptibility studies have been carried out in different species of mosquito for both larvae and adults derived from the Cauvery basin and its tributaries from Thalakaveri to Makedatu in Karnataka (Shetty, 2002*b*). Susceptibility studies for various classes of insecticides, including organochlorines, organophosphates, carbamates, synthetic pyrethroids and botanicals, have been carried out on three major mosquito vector species (Ghosh *et al.*, 2002; Kashyap and Shetty, 2011; Shetty *et al.*, 2006, 2007, 2010, 2012). The genetic basis of chlorpyrifos (Chandrakala and Shetty, 2006) and propoxur (Sanil and Shetty, 2010) resistance and their cytological basis (Shetty *et al.*, 2013) have been studied. The susceptibility status of *An. stephensi* to DDT, BHC, propoxur, malathion, fenthion and deltamethrin (Mukhopadhyay *et al.*, 1997) has been reported in India. *Anopheles stephensi* from southern Iran (Manouchehri and Yaghoobi-Ershadi, 1988), *An. pulcherrimus* from southeast Iran (Zahirnia *et al.*, 2002) and Russia (Sorokin *et al.*, 1991), and *An. sacharovi* from Iran (Yaghoobi-Ershadi *et al.*, 2001) have been found to be susceptible to the propoxur treatment.

Adults of *An. culicifacies* from Surat, India (Raghavendra *et al.*, 2010) and *Anopheles gambiae* from Nigeria have been found to be susceptible to propoxur (Olayemi *et al.*, 2011); *Anopheles maculipennis* from Turkey has been found to be resistant (Akiner, 2014). Larvae of *An. stephensi* and *Anopheles subpictus* from Gujarat and Rajasthan, India (Tikar

Table 6. Range, mean ± SD and confidence limits (CL) of LC_{50} and LC_{90} resistance ratios for chlorpyrifos and propoxur

	LC_{50}			LC_{90}		
Insecticides	Range	Mean ± SD	95% CL	Range	Mean ± SD	95% CL
Chlorpyrifos ($n = 32$)	1.26 (PUN-A) to 37.68 (GOA-A)	8.9526 ± 10.198	5.388–12.5167	1.19 (PBN) to 47.46 (GOA-A)	10.6484 ± 11.9642	6.4671–14.8292
Propoxur ($n = 32$)	1.34 (PDC) to 12.77 (JPN)	2.8361 ± 2.3066	2.0299–3.6422	1.13 (PDC) to 12.32 (JPN)	3.0720 ± 2.3443	2.2527–3.8913

et al., 2011) have been found to be tolerant to chlorpyrifos (0.025 mg/l). *Anopheles dthali* and *An. fluviatilis* from southern Iran (Hanafi-Bojd *et al.*, 2006) and *An. gambiae* from Bénin, West Africa (N'Guessan *et al.*, 2010) have been found to be susceptible to chlorpyrifos-methyl; *An. sinensis* from the Republic of Korea has been reported to be resistant to chlorpyrifos (Chang *et al.*, 2013). Mixed results from different parts of the world are a serious concern, and underline the importance of monitoring and understanding resistance levels and their mechanisms. From the present study, although some geographical strains showed resistance to both insecticides, *An. stephensi* was found to be more susceptible to propoxur than to chlorpyrifos. Baseline information from this experiment will serve as a guide for future application of insecticides to different strains of *An. stephensi*.

The first step is to assess trends in frequency of the resistance gene(s) through susceptibility tests, and to investigate the efficacy of insecticides using bioassays. The most effective insecticides giving 100% possible kill should be used in rotation in vector control programmes (Kasap *et al.*, 2000). Application of inappropriate insecticides without an understanding of the prevailing resistance mechanisms may lead to control failure. Hence, periodic monitoring of insecticide resistance status is an important criterion in vector control programmes (Shetty *et al.*, 2012). The rate at which an insecticide becomes ineffective depends on the selection pressure for resistance, which is determined by monitoring, and how often and for how long the insecticide is being used (Hudson, 1983).

Conclusion

The type form of *An. stephensi* was found in urban and semi-urban areas and the intermediate form in semi-urban localities. The forms are usually vectors, and are distributed throughout the urban localities in and around Bangalore. The different strains across India showed that they were more susceptible to propoxur than to chlorpyrifos. Hence, propoxur can be preferred and used ahead of chlorpyrifos. Close cooperation is necessary among health, agriculture and ecosystem analysts to understand insecticide use. The data provided in the bioassays provide the preliminary information important for subsequent investigations into the mechanisms of resistance.

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