Vector Biology and Control

1.1 Studies on *Anopheles* species Complex

1.1.1 *Anopheles culicifacies* Complex

Bionomics and distribution pattern

Analysis of *Anopheles culicifacies* populations from malaria-affected Gaurella and Pendra blocks of District Bilaspur (Chhattisgarh) revealed prevalence of species B and C with predominance of the latter. *An. culicifacies* C appeared to be the prime vector of malaria as it was found sympatric with *An. fluviatilis* T, a poor/non-vector in Khargone, Harda and Khandwa districts of Madhya Pradesh state. *An. culicifacies* samples collected during peak transmission season were analysed for sibling species composition and blood meal source. Only established vector species were encountered constituting 76.5% *An. culicifacies* and 23.5% *An. fluviatilis*. Both species were found predominantly zoophagic. Similarly, *An. culicifacies* examined from villages selected for malaria vaccine trial in and around Jabalpur (Madhya Pradesh) revealed the prevalence of species B, C and D with predominance of species C (62%). The established vector species (C and D) together constituted 90% of the total *An. culicifacies* population, while the prevalence of non-vector species was low (~10%) suggesting high malariogenic potential of the study area. In District Jalpaiguri (West Bengal), a few samples analysed from PHC Alipurdwar area showed the prevalence of *An. culicifacies* species B and C in almost equal proportion.

1.1.2 *Anopheles fluviatilis* Complex

Distribution, bionomics and biology of sibling species

In district Jalpaiguri of West Bengal, a malaria endemic district, *An. fluviatilis* was found sympatric with *An. minimus* in villages under PHCs Alipurdwar and Kalchini. Identification of the collected specimens to sibling species, using cytological technique and allele-specific PCR assay, revealed presence of *An. fluviatilis* species T. Blood meal source analysis using counter current immuno-electrophoresis showed species T to be totally zoophagic suggesting that the species might be playing a secondary/negligible role in malaria transmission in the district. Similarly, in Gaurella and Pendra blocks of District Bilaspur (Chhattisgarh) that witnessed spurt in malaria cases during October–November 2006, cytogenetic characterisation of *An. fluviatilis* population revealed the prevalence of species T which was polymorphic for q^1 inversion. In study villages, this species was found resting predominantly in cattlesheds and was totally zoophagic which indicates its limited role in malaria transmission in District Bilaspur.

1.1.3 *Anopheles minimus* Complex

Anopheles minimus collected from Districts Sonapur and Dibrugarh of Assam and District Jalpaiguri of West Bengal were characterised for Internal Transcribed Spacer 2 (ITS2) and 28S rDNA (D2-D3 domain) and based on these sequences, all were identified as species A (*An. minimus s.s.*). Molecular characterisation of morphologically identified *An. fluviatilis* collected from District Sonapur (Assam) revealed that their ITS2 and 28S rDNA are homologous to sympatric species *An. minimus s.s.* and are different than all reported members of the Fluviatilis Complex. Based on these findings, it was inferred that morphologically identified *An. fluviatilis* from Assam are, in fact, morphological variant (hypermelanic form) of *An. minimus s.s.* Cytogenetic characterisation of the *An. minimus s.s.* samples revealed striking resemblance in their banding

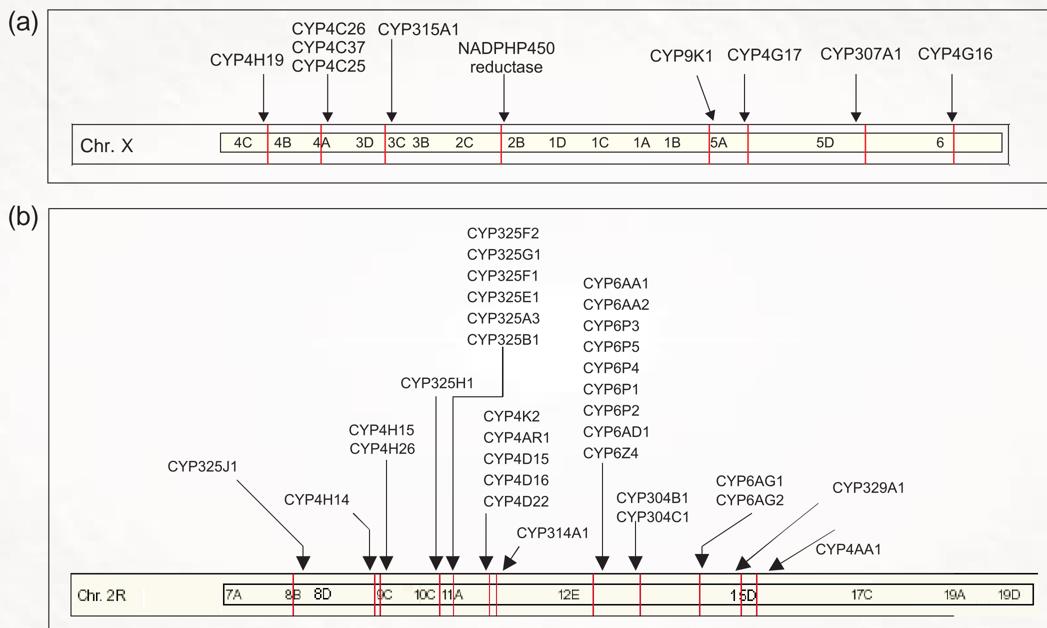
pattern of polytene chromosomes with that of *An. flvuiatilis* species U. However, molecular characterisation of these two species based on 28S (D2-D3) and ITS2 ribosomal DNA and cytochrome oxidase II (mtDNA) sequences revealed that these two species are genetically distant. A detailed photomap of polytene chromosomes complement of *An. minimus* is under preparation. *An. minimus* species A prevalent in hilly forested areas in districts of Assam and West Bengal was found resting predominantly in human dwellings. Blood meal source analysis revealed species A to be highly anthropophagic with anthropophilic index (AI) > 90%. These observations strongly suggest that *An. minimus* species A is playing a major role in malaria transmission in the study areas of the districts surveyed.

1.2 Vector Genomics

1.2.1 Genomic characterisation of cytochrome P450 genes in *Anopheles gambiae*

Origin and spread of insecticide resistance is an important example of natural selection, and the factors governing the maintenance of resistance-associated mutations are both of academic and of applied importance. The primary causes of insecticide resistance are: (i) alterations in the target sites; and (ii) increase in the rate of insecticide metabolism. Metabolic-based resistance mechanisms are important in conferring insecticide resistance and biochemical analyses have identified three enzyme families responsible in insecticide metabolism, the cytochrome P450s (P450s), GSTs (glutathione-S-transferases) and COE (carboxylesterases).

Cytochrome P450 (CYP) is an important and diverse superfamily of hydrophobic, haeme—containing enzymes involved in the metabolism of numerous endogenous and exogenous compounds. In the present work, genes of this superfamily were identified by fine scale scanning of all the four major chromosomal arms of the African malaria vector, *An. gambiae*, since the whole genome sequence of this species of *Anopheles* is available at the public domain. Scanning of the X, 2R, 2L, 3R and 3L chromosomes for CYP genes using the ENSEMBL (www.ensembl.org) genome browser database (release 43, February 2007) revealed a total of 83 CYP genes, clustered in different subfamilies found across the whole genome (Figs. 1a–e). Interestingly, as revealed from the figures, clustering of genes are seen at several places related to genes from a particular subfamily. Of particular importance,



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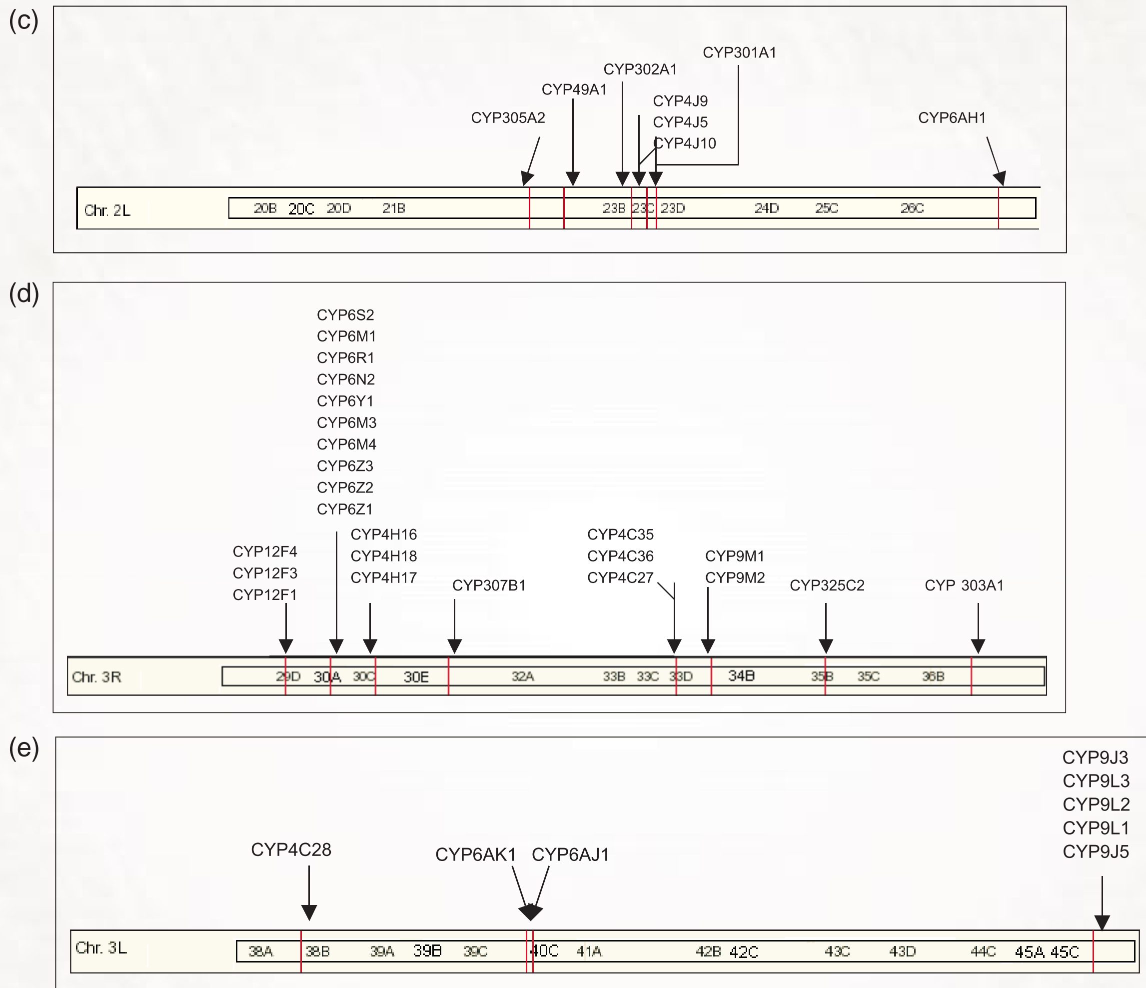


Fig. 1: **Schematic diagram showing the location of cytochrome P450 genes in (a) X- chromosome; (b) chromosome 2R; (c) chromosome 2L; (d) chromosome 3R; and (e) chromosome 3L of *An. gambiae***

two subfamilies, CYP6 and CYP4 are found to be present as clusters in whole genome, but CYP9 subfamily clusters are present only in chromosome 3L. In chromosome 3 most of the CYP genes are present at the telomere and proximal ends. In chromosome 2 the CYP genes are present at only the proximal ends. In contrast, most of the CYP genes are centrally located in the telocentric X-chromosome. The distribution of total CYP genes in each chromosomal arm is shown in Fig. 2 and the schematic diagram showing organisation of each CYP gene along with the sizes are shown in Fig. 3.

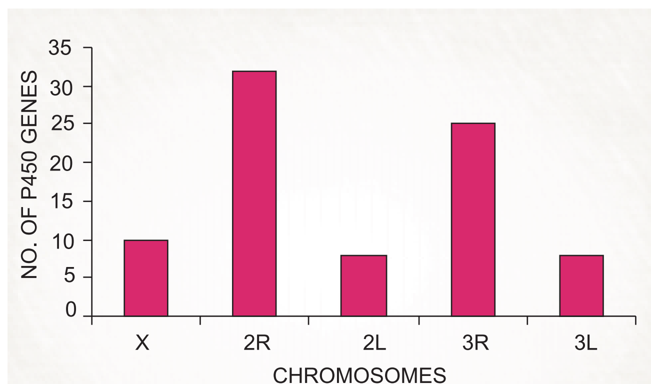


Fig. 2: **Distribution of cytochrome P450 genes in chromosomes of *An. gambiae***

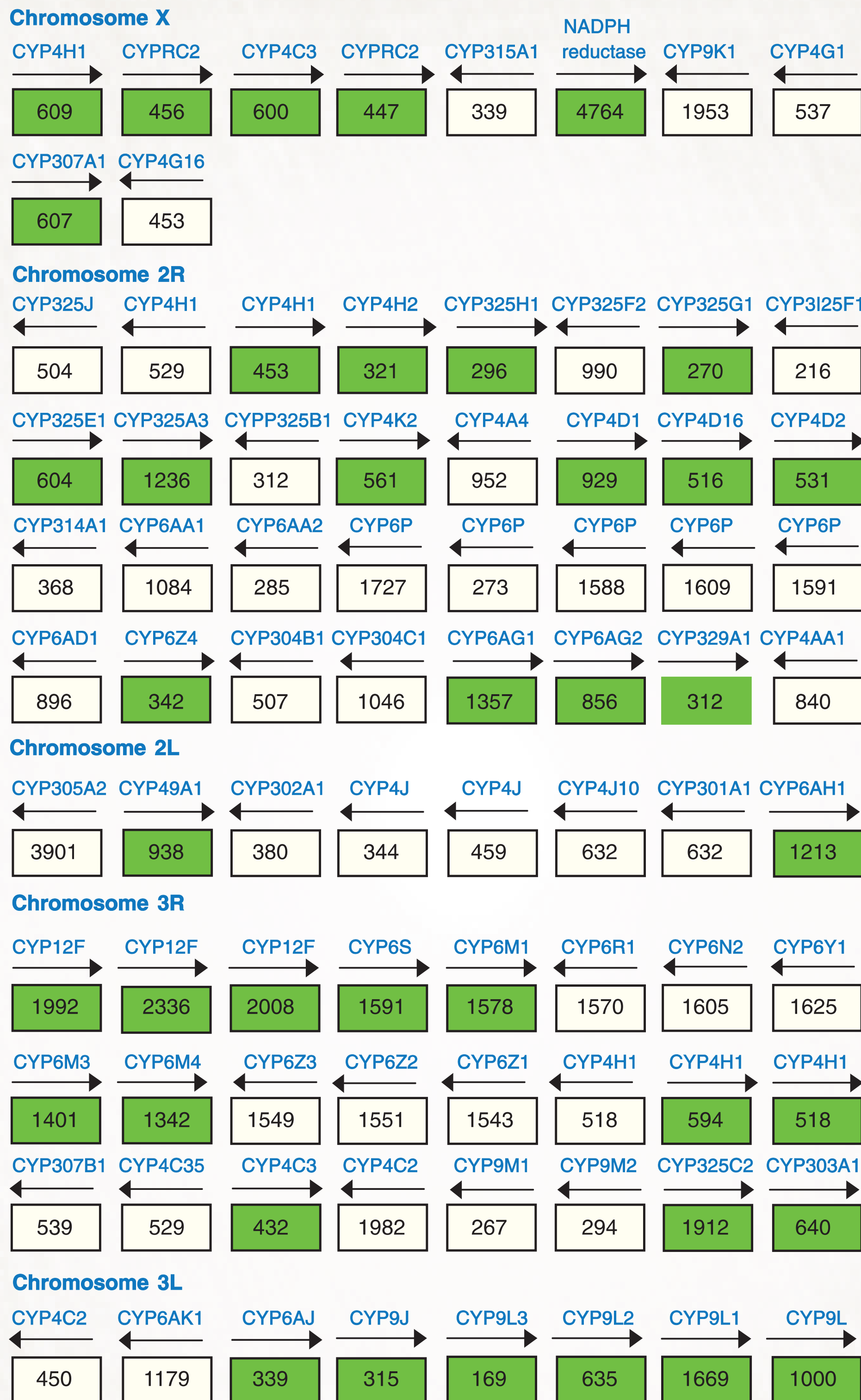


Fig. 3: **Schematic diagram showing the organisation of all 82 cytochrome P450 genes in different chromosomes of *An. gambiae*. Each solid rectangle represents a gene. Arrow marks show the directions of transcription. Green coloured rectangles denote forward and yellow coloured denote reverse transcription. Size (in nucleotide base pairs) of each gene is shown inside the rectangles (genes)**

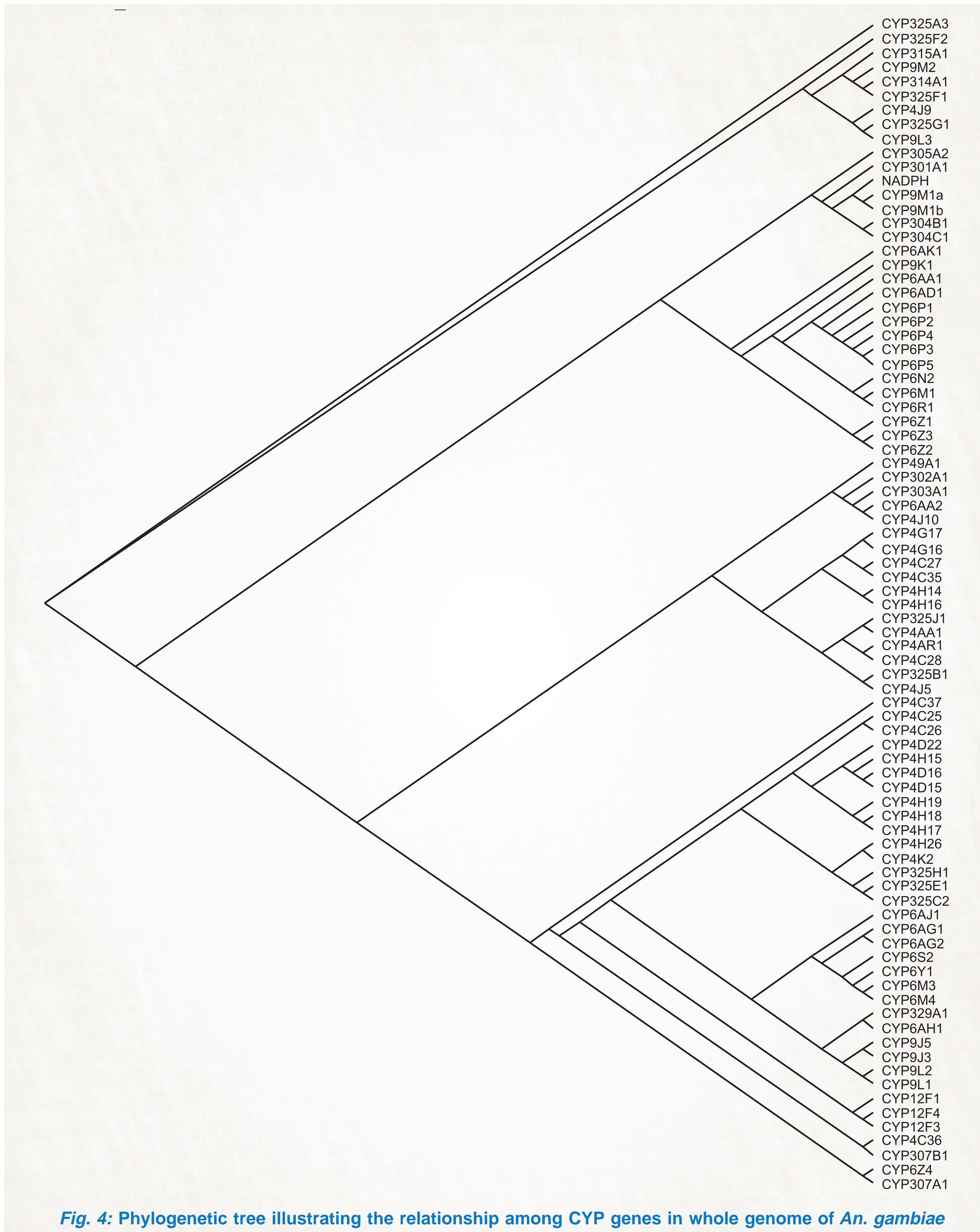


Fig. 4: **Phylogenetic tree illustrating the relationship among CYP genes in whole genome of *An. gambiae***

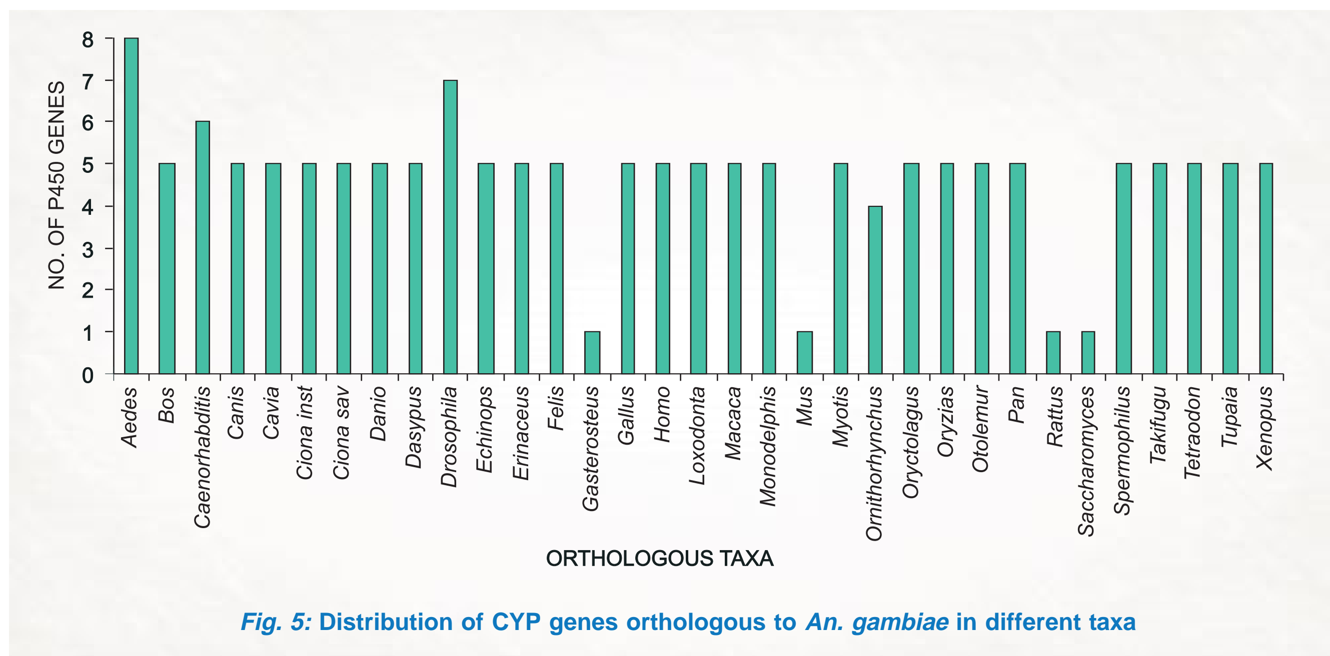


Fig. 5: **Distribution of CYP genes orthologous to *An. gambiae* in different taxa**

The nucleotide sequences of all the 83 CYP genes of *An. gambiae* were downloaded and multiple sequence alignment was successfully performed following ClustalX algorithm. The alignment is followed by construction of phylogenetic trees with the help of different softwares and bioinformatics tools. The trees show the evolutionary relationship among the CYP genes (Fig. 4). Interestingly, the CYP325A3 gene seems to behave like an out-group sequence and thus could have been very much similar to the ancestral sequence of the whole CYP gene families (Fig. 4). The secondary structures of all the genes are also predicted to know about the presence of helix, coils and extended loops.

In order to find if these genes have homologous sequences across taxa, we made a BLAST search in ENSEMBLE (release 44, April 2007) which revealed that *Aedes*, *Drosophila* and *Caenorhabditis* are genetically close at the CYP genes of *An. gambiae*. Nine CYP genes of *An. gambiae* were found to be conserved in >20 taxa and remaining were homologous in <10 taxa (Fig. 5). Interestingly, genes that are homologous to high number of taxa were found to be present in chromosome-X, followed by the right and left arms of the third chromosome.

The above evolutionary bioinformatic studies of cytochrome P450 genes in *An. gambiae* provide greater insights into genomic characterisation of insecticide resistance genes in the genus *Anopheles*. This information would be of tremendous importance to study the vectors of local importance in India such as *An. culicifacies*, *An. fluviatilis*, *An. minimus*, etc. Since the centrally and proximally located genes are expected to show high recombination, genetic variations in these genes are expected to me more. Thus, a comparison of genetic diversity among genes located in different regions of the genome in the above species of importance to India could be achieved through population genetic studies, which would ultimately lead to vector control strategy.

1.3 Vector control

1.3.1 Phase-I evaluation of chlorfenapyr (pyrrole insecticide) against susceptible and resistant strains of mosquito species

Chlorfenapyr, a pyrrole group pro-insecticide, acts by inhibiting the reaction of conversion of mitochondrial ADP to ATP. The observed diagnostic time of exposure and recovery period was determined.

An. culicifacies species A, species C, *An. stephensi* and *Cx. quinquefasciatus* were exposed to different concentrations (0.25–5%) of the insecticide for 15 to 180 min with recovery periods of 24, 48 and 72 h. Exposure of 120 min and recovery period of 48 h was needed to determine the susceptibility. The study is in progress.

1.3.2 Bioefficacy of "Advanced Odomos" repellent cream against vector mosquitoes with particular reference to *Anopheles stephensi*, malaria vector and *Aedes aegypti*, vector of dengue and DHF

This study was carried out to evaluate the bioefficacy of "Advanced Odomos" repellent cream against vector mosquitoes with particular reference to *An. stephensi* and *Ae. aegypti* under laboratory conditions and to determine average protection time against target mosquito species. DEET cream was used as positive control. Laboratory evaluation of Odomos repellent cream showed slightly higher protection time against *An. stephensi* than *Ae. aegypti* but the difference was not significant. The efficacy of Odomos cream in repelling the mosquitoes was comparable with 12% DEET cream. Exposure of mosquitoes up to 4 h on human skin (hand) applied with Advanced Odomos @ 1–10 mg/m^2 in a cage, produced 51.6–100% protection against *An. stephensi* and 55.1– 96.5% protection against *Ae. aegypti*, whereas exposure to DEET cream applied at the same dosages, i.e. 1–10 mg/m^2, produced 57.3–100% protection against the bites of *An. stephensi* and 59.9– 97.2% protection against *Ae. aegypti* (Tables 1–2). Application of Odomos cream on human skin at 10 mg/cm^2 gave 100% protection up to 4 h against *An. stephensi* but 100% protection was obtained at 12 mg/cm^2 in the case of *Ae. aegypti*.

Field trials of Advanced Odomos vs. DEET cream against malaria vectors were carried out in Pechera village in Loni PHC, District Ghaziabad, while field trials against *Ae. aegypti* were carried out during day-time in Railway Colony, Badarpur, Delhi. Results of field trials of Advanced Odomos vs DEET cream during whole night bait collections against different species of mosquito showed that percent repellency with Odomos cream applied at 4–10 mg/cm^2 ranged between 80.5 and 100% against *An. culicifacies*; and 70.5 and 100% against *An. stephensi*. Average protection time of 5.7–11 h against *An. culicifacies*, and 5–11 h against *An. stephensi* was observed. The percent repellency with DEET cream applied at the same dosages varied between 72 and 100% against *An. culicifacies*, 76.4 and 100% against *An. stephensi* and average protection time of 6–11 h against *An. culicifacies*, and 6.5–11 h against *An. stephensi* was observed (Table 3). The results revealed no considerable difference in the percent repellency of Odomos and DEET cream against the two malaria vector species tested.

TABLE 1

Laboratory evaluation of Advanced Odomos and DEET creams against *An. stephensi* and *Ae. aegypti* in cage bioassays

Dose (mg/cm^2)	% protection			
	An. stephensi		*Ae. aegypti*	
	Odomos	DEET	Odomos	DEET
1	33.7 (0)	45.0 (0)	32.5 (0)	39.7 (0)
2	62.7 (1)	69.2 (1)	56.2 (0)	72.5 (1)
4	77.0 (1)	81.8 (3)	72.0 (1)	85.5 (2)
8	100.0 (>4)	100.0 (>4)	97.9 (3)	98.0 (4)

Repellent cream was applied on nylon nets covered on Petri dish containing soaked cotton in glucose; Figures in parentheses are percent protection in hours.

TABLE 2

Laboratory evaluation of the repellent action of Advanced Odomos and DEET creams against *An. stephensi* and *Ae. aegypti* in cage bioassays (human exposure test)

Dose (mg/cm^2)	% protection			
	An. stephensi		*Ae. aegypti*	
	Odomos	DEET	Odomos	DEET
1	51.6 (1)	57.3 (1)	55.1 (0)	59.9 (0)
2	56.0 (1)	61.2 (1)	59.3 (1)	62.7 (1)
4	84.7 (1)	88.0 (2)	76.1 (0)	86.9 (0)
8	95.5 (2)	97.0 (2)	87.5 (2)	90.2 (2)
10	100.0 (4)	100.0 (4)	96.5 (4)	97.2 (3)
12	–	–	100.0 (4)	100.0 (4)

Figures in parentheses are protection time in hours.

TABLE 3

Field evaluation of Advanced Odomos cream against malaria vectors during whole night biting collection

Vector species	Repellent	Dose of repellent (mg/cm²)	No. of mosquitoes landed E/C	(%) Protection	Average protection (hours)
An. culicifacies	Odomos	4	7/36	80.5	5.7
		8	8/41	80.4	9.5
		10	0/37	100	11
	DEET	4	10/36	72.2	6
		8	6/41	85	9.1
		10	0/37	100	11
An. stephensi	Odomos	4	10/34	70.5	5
		8	18/100	82.5	8.1
		10	0/51	100	11
	DEET	4	8/34	76.4	6.5
		8	9/100	91	8.8
		10	0/51	100	11

Data of eight replicates; E—Experimental (Advanced Odomos cream); C—Control (Cream without any repellent ingredient).

The results of the field trials against *Ae. aegypti* mosquitoes showed 36.9 to 92.5% protection (repellency effect) at 4–10 mg/cm² application rate of Odomos cream and 39.1 to 96.2% protection at 4–10 mg/cm² of DEET cream (Table 4). Odomos cream considerably reduced the *Ae. aegypti* biting at 8 and 10 mg/cm² doses, as it resulted in 4 to 6 h protection at 8 and10 mg/cm². DEET cream produced 100% protection up to 5 and 6 h at the same doses. At lower dose of 4 mg/cm² Odomos cream failed to show any repellent effect against *Ae. aegypti* biting, whereas DEET cream gave repellency effect for one hour.

TABLE 4

Field evaluation of Advanced Odomos and DEET creams against *Ae. aegypti* during day time biting collection

Repellent	Dose of repellent (mg/cm²)	No. of mosquitoes biting E/C	(%) Protection	Average protection (hours)
Odomos	4	29/46	36.9	0.7
	8	7/64	89.0	4.7
	10	4/54	92.5	6.2
DEET	4	28/46	39.1	1.2
	8	5/64	92.1	5.2
	10	2/54	96.2	6.75

Data of four replicates; E—Experimental (Repellent cream); C—Control (Cream without any repellent ingredient).

All the volunteers participated in the study did not report any adverse effects such as itching, irritation, pungent smell, and other aberrations of the skin. Odomos cream applied on exposed body parts with 10 mg/cm² concentrations provided 100% protection from *An. culicifacies* for 11 h. Similarly, the protection time from the bites of *An. stephensi* for the same dose was 11 h. The present study also found that the Advanced Odomos cream when used in similar dosage on volunteers during day-time showed complete protection for 6 h against *Ae. aegypti*.

In conclusion, the efficacy of Advanced Odomos cream is comparable to the known repellent cream DEET for protection against the bites of malaria and dengue vectors.

1.3.3 Evaluation of ZeroFly®, an insecticide incorporated plastic sheeting against mosquitoes with particular reference to malaria vectors

This study on the efficacy of ZeroFly® plastic sheeting (incorporated with deltamethrin @ 265 mg a.i./m²) against malaria vectors, sponsored by M/s. Vestergaard Frandsen India Pvt. Ltd., New Delhi, was initiated in the month of July/August 2006 in labourer camps in Delhi and Noida and in the month of December in RAC police camp in Delhi.

Fig. 6: **Temporary shelter in a labourer camp made with ZeroFly**

In all these localities ZeroFly plastic sheets and untreated plastic sheets were fixed in tents (Fig. 6) at a distance of at least 1 km (Control). Entomological and epidemiological parameters were monitored at fortnightly intervals. The results showed considerable reduction in the indoor resting density of vector and non-vector insects in the labourer camps provided with ZeroFly plastic sheeting, as compared with the control plastic sheetings. In a labourer camp at a construction site in Noida, the indoor resting man hour density (MHD) of anophelines was in the range of 0–1.5 in the experimental area as compared to 1.5–232 in the control area. Similarly, in JJ cluster inhabiting agricultural labour in the Yamuna River belt area near Madanpur Khader in Delhi, the indoor resting MHD of anophelines was in the range of 0–5 in the experimental area as compared to 2–248 in the control area (Fig. 7). Slide positivity rate (SPR) in the construction site of labourer camp in Noida was 0–5 in the experimental area as compared to 10–33.3 in the control area. Similarly, in the JJ cluster in Delhi the SPR in experimental and control areas was 0 and 10–22.2. In RAC police camp preliminary collections revealed the reduction of culicine density in the experimental tents as compared to the control tents. In addition, evaluation of ZeroFly was also being undertaken in tribal hamlets in Orissa. The study is in progress.

1.3.4 Follow-up study on the efficacy of Olyset®Nets against malaria vectors and on malaria transmission in District Gautam Budh Nagar (Uttar Pradesh)

Follow-up studies were initiated in three villages to study the long-lasting efficacy of Olyset nets. Olyset nets and untreated nets were distributed in August 2004 in Khandera and Beel Akbarpur villages, respectively and Anandpur village was kept as control where nets were not used. Fortnightly monitoring of the MHD of mosquitoes and surveillance of malaria incidence was carried out. Results revealed a remarkable difference in the indoor resting density of mosquitoes particularly the major malaria vector *An. culicifacies* in the Olyset-

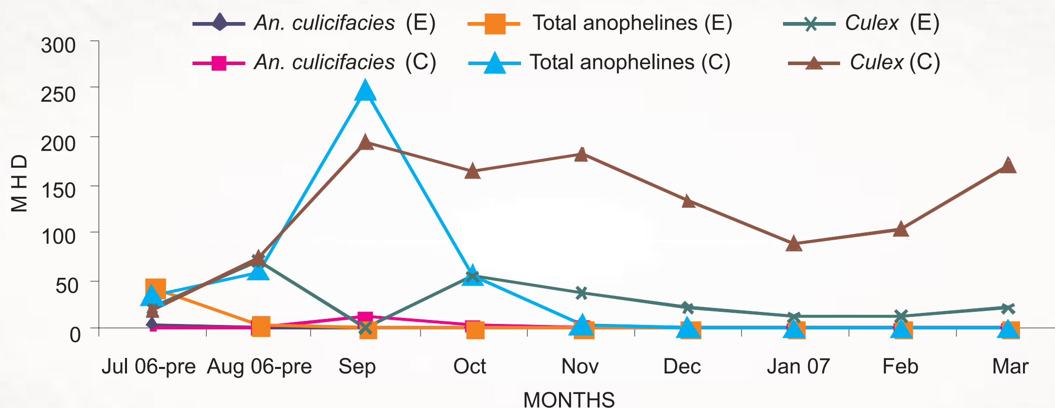


Fig. 7: **Indoor resting density of mosquitoes in experimental (ZeroFly) and control (Untreated plastic sheets) structures in a labourer camp in Delhi**

net village when compared to the untreated net and no net villages. The mean MHD of this species was 6.9 in human dwellings with Olyset net as compared to 19.2 and 21.9 in untreated net and no net villages respectively. Epidemiological results from the three villages revealed considerable difference in the prevalence of malaria. Cases/000 was nil in village with Olyset net as compared with 3.8 and 15 in plain net and no net villages respectively. Similarly, Pf/000 in the three villages was 0, 0.5 and 5.5 respectively. Results revealed that use of Olyset nets resulted in complete interruption in malaria transmission and also reduced mosquito nuisance. The study is still in progress.

1.3.5 Evaluation of Bacticide

Results of the simulated trial with Bacticide tablets in plastic containers revealed 100% mortality of *Ae. aegypti* larvae in small containers of 10 and 50 litres but in bigger containers of 100 litres of water treated with one tablet, there was only 90% mortality in *Ae. aegypti* larvae and the remaining larvae got pupated and emerged as adult mosquitoes. The tablet was more effective in smaller containers of ~10 to 50 litres as it produced 100% reduction in the density of *Ae. aegypti* after 2 to 3 days and this impact persisted up to 2 weeks of observation. However, in tanks of ~100 litres or more, the tablets did not result in 100% reduction in the density of III and IV instar larvae.

1.3.6 Evaluation of mosquito larvicide Temephos for use in polluted water

This study was initiated in the month of January 2007 to evaluate the efficacy and persistence of the larvicide Temephos 50% EC in different types of polluted water at different application rates (25, 50,100 and 200g a.i./ha doses) in three different urban areas in India. The larvicide was applied with the help of a Knapsack sprayer at the rate of 20 ml/m^2 after making required dilutions for different doses. The impact of larvicide was assessed by monitoring the immature density before and after application of larvicide at different time intervals on Day 1, 2, 3, 7, 10, 14 and then at weekly intervals and determining the percent reduction based on untreated control. The study is in progress.

1.3.7 Studies on the adulticidal activity of *Fusarium pallideroseum* and *Aspergyllus nidulans* isolated from adult *Culex quinquefasciatus* in Delhi

Studies were carried out to isolate, identify and determine natural entomopathogenic fungi from field collected moribund adult *Cx. quinquefasciatus*, and their mosquitocidal activity against adult *Cx. quinquefasciatus*. Two fungal species *Aspergyllus nidulans* var *acristatus* (ATCC-6327.04) and *Fusarium pallideroseum* (ATCC-6324.06) were isolated. Adult bioassays were carried out using spore impregnated papers essentially following WHO susceptibility test method. *F. pallideroseum* was found to be more effective compared to *A. nidulans* var *acristatus*. About 90% mortality was observed after 4 h exposure to *F. pallideroseum* spore impregnated paper at a concentration of 1.11x 10^{10} spores/m^2.

1.3.8 Induction of chymoelastase (Pr1) of *Metarhizium anisopliae* and its role in causing mortality in mosquito larvae

Three isolates of insect pathogenic fungus, *Metarhizium anisopliae* produced extracellular cuticle-degrading enzymes chymoelastase (Pr1) and trypsin like protease (Pr2) in variable amounts. Induction of both Pr1 and Pr2 was directly proportional to the incubation time of different carbon and nitrogen sources and maximum inducer was from mosquito cuticle. The induction of Pr1 was found to be higher in the *M. anisopliae* 892 strain than in the strains 3210 and 4102 [Strains obtained from MTCC]. The larvae of *Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti* were susceptible to different strains of *M. anisopliae* in the following order: strain 892>3210>4102. *Cx. quinquefasciatus* was the most susceptible. The cuticle of *Cx. quinquefasciatus* has induced maximum Pr1 than the rest of the mosquito cuticles. A direct relation between the quantum of induction of Pr1 and mortality was observed. Among the cuticles of four *Anopheles* spp added basal medium, highest induction of Pr1 was observed in *An. culicifacies* species C and lowest with *An. fluviatilis*. Pr2 induction of *M. anisopliae* did not correlate with the mortality of mosquitoes.

1.3.9 Micropropagation of an antimalarial plant (*Spilanthes acmella* L.) and investigation for larvicidal efficacy

Micropropagation has been achieved for a promising larvicidal asteraceous taxon *Spilanthes acmella* L. using seedling leaf explants and reared on a variety of growth regulators. Bioassays were conducted with crude extract in hexane from different plant parts of *in vitro* regenerated plants such as flower head, leaf, stem and roots, and the toxicity was in the order of flower>leaf>stem, and root was most effective against *An. stephensi* and *Cx. quinquefasciatus*. The LC_{50} with root extract was 2.71 ppm (*An. stephensi*) and 1.19 ppm (*Cx. quinquefasciatus*). The work for chemical characterisation of the compound responsible for lethality is in progress.

1.3.10 Bioefficacy of herbal extract from flower heads of *Spilanthes acmella* L.

Hexane extracts of flower head of three species of the Genus *Spilanthes: S. acmella* L. var *oleracea* Clark, *S. calva* and *S. paniculata* Wall. ex DC were tested for their efficacy against *An. stephensi*, *An. culicifacies* and *Cx. quinquefasciatus* at different concentrations in the range of 1.5625–50 ppm. The LC_{50} and LC_{90} for different species against *An. stephensi* is 4.57 and 7.83 ppm (*S. acmella* L. var *oleracea*), 5.1 and 8.46 ppm (*S. calva* L.) and 5.09 and 13.09 ppm (*S. paniculata* Wall. ex DC), respectively. This is the first report on the larvicidal activity of *S. calva* and *S. oleracea*.

1.4 Insecticide resistance

1.4.1 Insecticide resistance studies in Surat (Gujarat)

Field studies were carried out in the villages in Ukalda PHC area, District Surat in November 2006. Studies carried out in November 2005 and in the present study, *An. culicifacies s.l.* populations have indicated complete reversal of pyrethroid resistance reported in 2002. In these villages insecticide spray was withdrawn about 5–6 years back. Mosquitoes were exposed to the designated WHO diagnostic doses of different insecticides. This species was found resistant to DDT and malathion, while it was susceptible to other organophosphates (fenitrothion 100%), carbamates (propoxur 96%; bendiocarb 99%) and pyrethroids (deltamethrin 99%; lambdacyhalothrin 97%; cyfluthrin 97% and permethrin 96%). There was no variation in the susceptibility of this species in studies carried out in 2005 and 2006 except in DDT and malathion. As observed in earlier studies in 2005, *An. culicifacies* had shown narrow spectrum of resistance and the synergistic studies have confirmed involvement of malathion carboxylesterase (MCE) for conferring malathion resistance.

1.4.2 Study on the susceptibility of *Culex* larvae to Fenthion in urban localities of Delhi

The efficacy of Fenthion against *Cx. quinquefasciatus* larvae collected from urban areas of Delhi from June 2006 to January 2007 was studied in the laboratory conditions using WHO discriminatory dose. The field evaluation of Fenthion was carried out in waste water collections and in ditches of four localities of Delhi—Harsh Vihar (east zone), Ranaji Enclave (west zone), Kadi Vihar (north zone) and Roop Nagar (south zone). Pre-treatment baseline larval/pupal densities were recorded in each habitat using standard larval dippers. In drains, 10 dips were taken at every 5-metre distance for measurement density of *Culex* immatures. Similarly, 5 dips were taken from each of the pits/ditches.

National Vector Borne Disease Control Programme (NVBDCP) recommends mixing 5 cc of Fenthion 100% EC formulation with 10 litres of clean water for spraying in mosquito breeding habitats using a Knapsack sprayer @ 20 cc/m^2. After pre-treatment recording of larval density, spraying was done in experimental habitats while the unsprayed habitats were used as control for comparison. Four replicates of experimental and 2 replicates of control habitats were selected. The larval densities were recorded in all the Fenthion-treated and control habitats on Day 0 (pre-treatment) and thereafter on Days 1, 3, 5 and 7 post-treatment. Second and third applications of Fenthion were made at weekly intervals and larval density was monitored. The

reduction in III/IV instar larvae was calculated using Mulla's formula.

To evaluate susceptibility/resistance of *Cx. quinquefasciatus* larvae to Fenthion, III/IV instar larvae were collected from trial habitats and brought to the laboratory. Such larvae were exposed to Fenthion at the diagnostic concentration of 0.125 ppm (as well as two serially lower concentrations of 0.05 ppm and 0.025 ppm) in the laboratory. One ml of the Fenthion stock solution supplied by NIMR HQs was added in 249 ml of the water in beakers. Insecticide was added just below the surface of the water and stirred. At least three replicates of 25 larvae each for test and concurrent controls with two replicates of 25 larvae were run. After 24 h of exposure, mortality was determined scoring the dead and moribund larvae. Larvae, which pupated, were discarded and counted for calculation of mortality. Corrected percent mortality was calculated using Abbott's formula when the larval mortality in control was between 5 and 20%.

Field evaluation

The results indicate that spray by Fenthion resulted in reduction of larval density up to a maximum of 72.2% on Day 1 in waste water collections. Reduction was achieved only up to third day of spray. In ditches also, the reduction in larval density was observed up to third day after spray and the maximum percent reduction was 90.6 on Day 1. Thereafter there was no reduction in larval density and on the fifth day the density rather increased and percent reduction values were in negative. Even second and third rounds of spray did not result in improvement in percent reduction after third day. In all the four sites in Delhi, the larval density in experimental habitats on Day 21 was more than that of on Day 0.

Laboratory evaluation

The results of laboratory evaluation on susceptibility of *Culex* larvae to different concentrations of Fenthion are given in Table 5. It was found that there was 89.5% mortality at 0.125 ppm, 53.4% at 0.05 ppm and 40.9% at 0.025 ppm.

It can be concluded that the spraying of Fenthion at the NVBDCP recommended dose produced a very low impact on larval densities in general. The effect lasted only up to the third day. The gradual rounds of spray resulted in further reduction in larval densities. On Day 21 of post-spray, the larval density was more than that of on Day 0, indicating no impact of larvicide. The results of the larval susceptibility tests on *Cx. quinquefasciatus* indicated that the corrected percent mortality in larvae at the dose of 0.05 ppm was 53.4% which revealed that *Cx. quinquefasciatus* had developed resistance to Fenthion. In Delhi, Fenthion is in operation for the last 20 years.

TABLE 5

Susceptibility of *Cx. quinquefasciatus* III and IV instar larvae to Fenthion under laboratory conditions in Delhi

Concentration (in ppm)	No. of replicates	No. of larvae exposed	Corrected % Mortality
0.125	4	100	89.5
0.05	4	100	53.4
0.025	4	100	40.9
Control (for each concentration)	2	50	6–14

1.5 Vector-parasite interactions

1.5.1 Studies on *Plasmodium*-refractory *An. culicifacies*

Differential expression of serine protease gene

Serine protease (acsp30) gene of *Plasmodium vivax*-refractory *An. culicifacies* was found to be *Plasmodium* inducible. Considerable differences were noticed between the sequences of the promotor region of refractory (R) and susceptible (S) strains, with ~1.5 fold higher promotor strength in the R strain. The increase in promoter activity in the R strain was attributed to sequence differences within the 400 bp region spanning –333 to –702 bp upstream of the start codon. To determine the effect of such differences on the binding of nuclear proteins, electrophoretic mobility shift assays (EMSAs) were performed. EMSA experiments with three different probes (400, 188 and 100 bp)

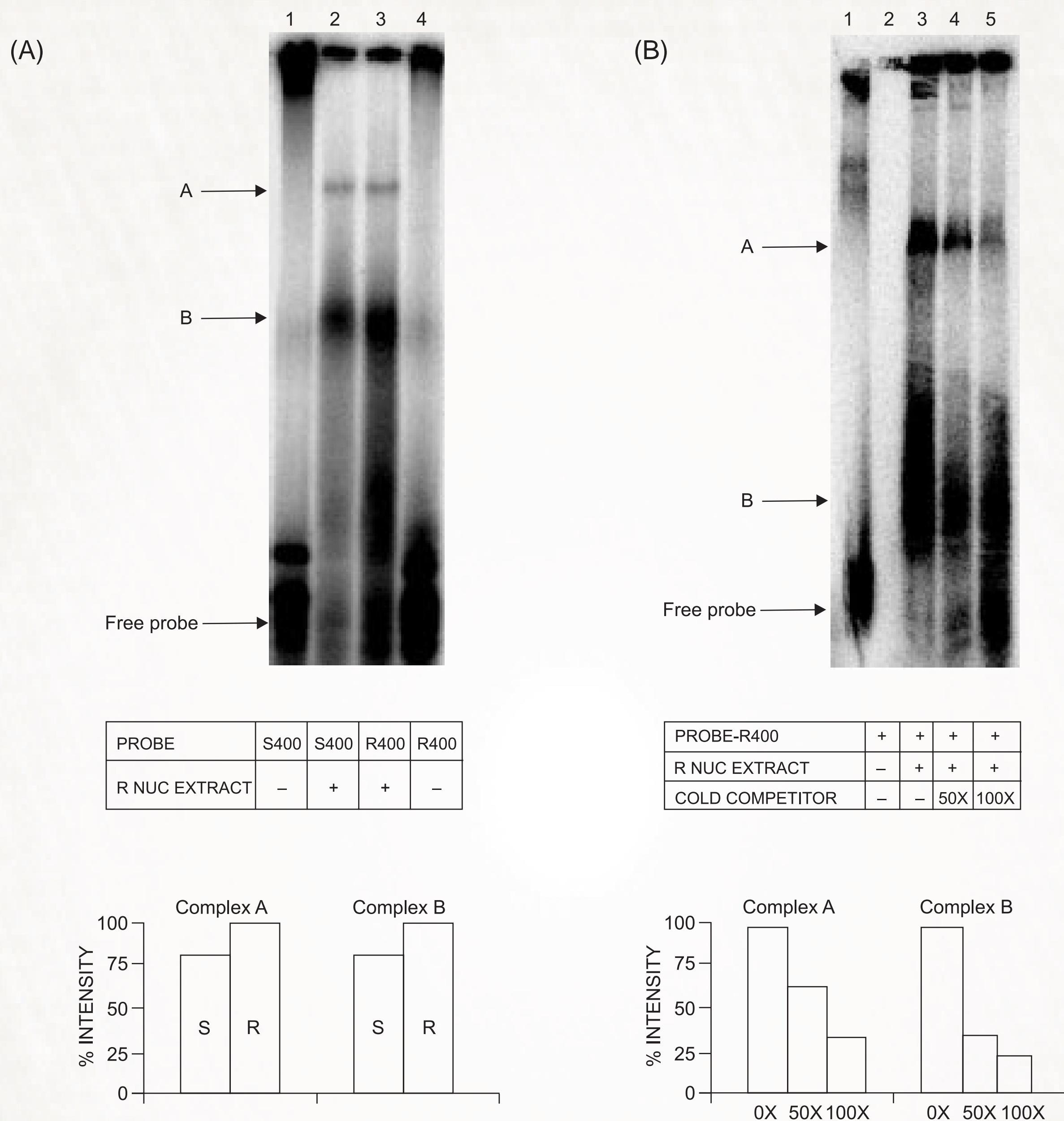
allowed us to determine the minimum upstream region that shows difference in binding of nuclear proteins and thus might be responsible for differential expression of acsp30 in the two strains. EMSAs using nuclear extracts from the R strain and R400/S400 probe revealed two complexes, a sharp slow migrating band, complex A and a faster moving diffused band, complex B (Fig. 8A, lane 3). Both the complexes were observed when R188 probe was incubated with nuclear extract from R strain (Fig. 9B, lane 3). Interestingly, formation of complex B was nearly abolished on R100 probe but complex A formation remained unaffected (Fig. 10B). These results clearly indicated that the 88 bases (–602 to –514 bp) missing in R100 probe were critical for the assembly of transcription factors forming complex B but the 100 bp upstream region (–702 to –602 bp) was important and sufficient for binding of nuclear proteins forming complex A. In general, the binding of nuclear proteins to probes derived from the S strain was less compared to that from the R strain, which further emphasised the importance of differences in upstream regions of acsp30 from both the strains. The greater intensity of the bands with the R probes showed that the formation of both the complexes was more on R probe than S; an approximately 25% increase in DNA binding activity of both the complexes was observed on R400 as compared to S400 probe. Importantly, when S188 was used as a probe, there was an approximately 50% reduction in the formation of complex B than on R188. This could be a consequence of an increase in sequence divergence (70%) in this region between the two strains. Specificity of interaction of nuclear proteins with various probes from R and S strains was evaluated by competition assays in the presence of corresponding specific cold probe. The binding of nuclear factors to 400 bp upstream sequence from R strain (R400) was highly specific as the formation of complexes A and B was reduced to 25% in the presence of 100-fold molar excess of unlabelled 400 bp cold probe in the EMSA binding reaction mixture (Fig. 8B). Competition experiments with sequentially shorter fragments from R and S strains also generated similar results showing the specificity of binding of nuclear factors to all the probes.

We also performed EMSA experiments using nuclear extract from both the strains to evaluate the presence of additional transcription factors in the R strain that could be absent in the S strain. Noticeably, the binding pattern of nuclear proteins from S strain to S188 and R188 probes (Fig. 9A) were different from that of nuclear proteins from R strain. A similar result was obtained with S100 and R100 probes (Fig. 10). When a nuclear extract from the S strain was used with R100 probe, the faster migrating band, complex B did not form. This is indicative of either a lack of the transcription binding factors in the S strain that form complex B or their low concentrations that prevent detection. The association of putative binding factors was quantified by converting intensity of signals to numerical values by using the Image Analysis Software, ImageQuant TL (Amersham Biosciences) and the results were presented as bars in Figs. 8, 9 and 10.

Analysis of the Phenoloxidase enzyme

Third and fourth instars male/female larvae of refractory strain were taken separately from same batch of mosquitoes in sodium phosphate buffer pH 6.8. Similarly, male and female pupae were also taken at 0 and 24 h of pupation, these were labelled as fresh and old pupae respectively. Prior to Phenoloxidase (PO) assay protein in the samples was estimated quantitatively using Bradford method. Quantitative PO assays were carried out using tyrosine as substrate. The end product was read at wavelength of 420 nm. Qualitative PO assays were carried out on 5% SDS-PAGE gels. Enzyme activity was visualised on gels using tyrosine for monophenoloxidase (MPO) and DOPA for diphenoloxidase (DPO).

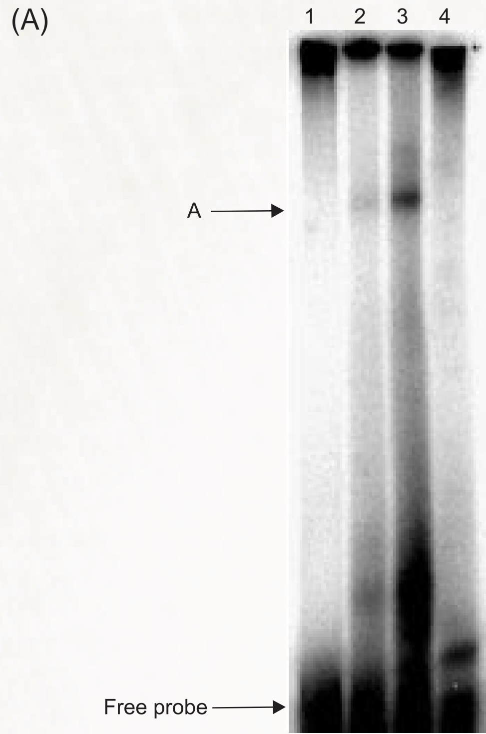
Quantitative assay revealed higher enzyme activity in IV instar than III instar larvae with highly significant difference. Similarly, older pupae had higher PO expression than the freshly developed pupae. Enzyme activity was nearly 3–4 times higher in larvae than pupae. Females of both III and IV instar larvae had significantly higher PO activity than the males. However, the PO activity in freshly emerged female pupae was nearly equal to freshly emerged male pupae whereas in older female

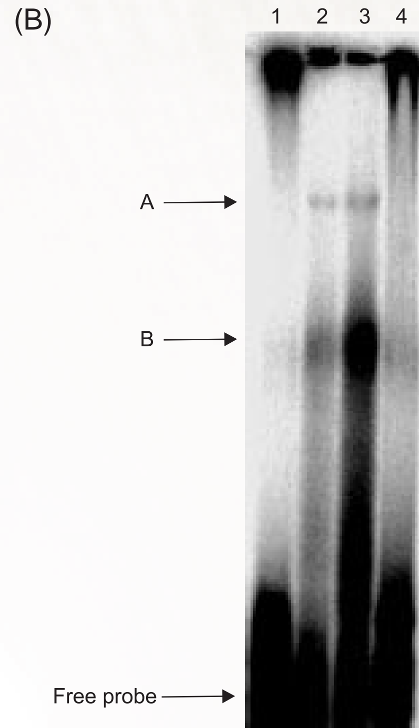


PROBE	S400	S400	R400	R400
R NUC EXTRACT	–	+	+	–

PROBE-R400	+	+	+	+
R NUC EXTRACT	–	+	+	+
COLD COMPETITOR	–	–	50X	100X

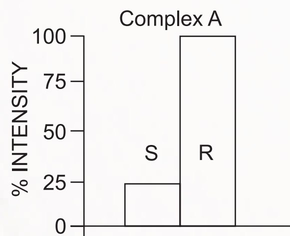
Fig. 8: **EMSAs with *An. culicifacies* refractory (R) strain nuclear extract and 400 bp probes from upstream sequences of acsp30 of S and R strains. (A) EMSA showing the binding pattern of nuclear proteins extracted from body tissue of five-day old adult female R strain mosquitoes when incubated with S400 (lane 2) and R400 (lane 3) probes at 37°C for 25 min. Free probes were run in lane 1 (S400) and lane 4 (R400); and (B) The radiolabelled probe R400 was incubated with refractory nuclear extracts without competitor (lane 3) and in the presence of unlabelled probe at 50-fold (lane 4) and 100-fold (lane 5) molar excess. Arrows indicate the migration of complex A (slow migrating) and B (fast migrating). The intensities of the signals were quantified with respect to PhosphoImager signals by the Image Analysis Software, ImageQuant TL (Amersham Biosciences) and represented as the percentage ratio of S400 to R400 signal intensities and as percentage ratio to the non-competed (R400) signal intensities**





PROBE	S188	S188	R188	R188
S NUC EXTRACT	–	+	+	–

PROBE	S188	S188	R188	R188
R NUC EXTRACT	–	+	+	–



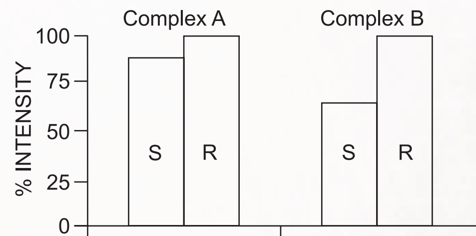
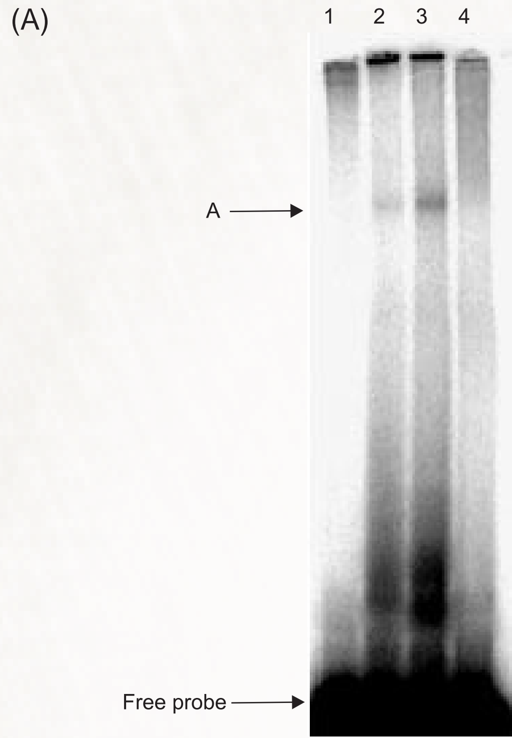
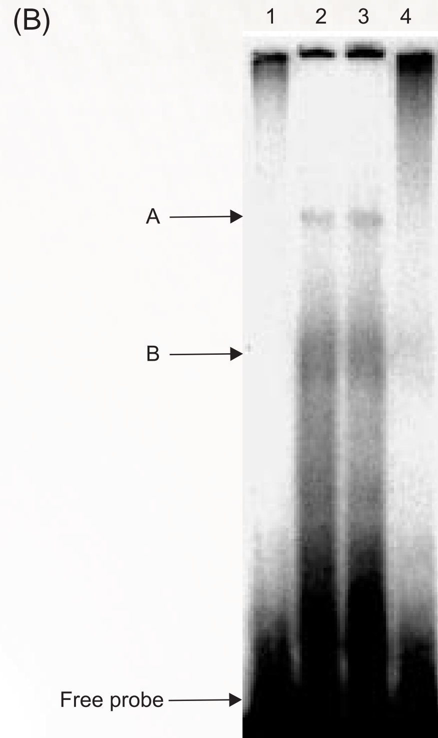


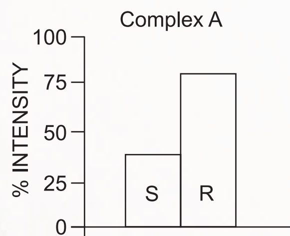
Fig. 9: **EMSAs using nuclear extracts and 188 bp probes from acsp30 upstream sequences. Nuclear proteins were extracted from body tissue of 5-day old adult female of S strain (A) and R strain (B) and incubated with S188 (lane 2) and R188 (lane 3) probes. Free probes were run in lane 1 (S188) and lane 4 (R188). Arrows indicate the migration of complex A (slow migrating) and complex B (fast migrating). The intensities of the signals were quantified with respect to PhosphoImager signals by the Image Analysis Software, ImageQuant TL (Amersham Biosciences) and represented as the percentage ratio of S188 to R188 signal intensities**





PROBE	S100	S100	R100	R100
S NUC EXTRACT	–	+	+	–

PROBE	S100	S100	R100	R100
R NUC EXTRACT	–	+	+	–



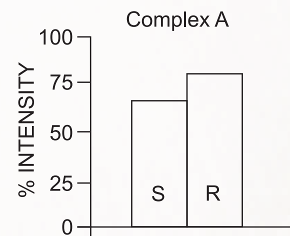


Fig. 10: EMSAs using nuclear extracts and 100 bp probes from acsp30 upstream sequences. Nuclear proteins were extracted from body tissue of 5-day old adult female of S strain (A) and R strain (B) and were incubated with S100 (lane 2) and R100 (lane 3) probes. Free probes were run in lane 1 (S100) and lane 4 (R100). Arrows indicate the migration of complex A (slow migrating) and complex B (fast migrating). The intensities of the signals were quantified with respect to PhosphoImager signals by the Image Analysis Software, ImageQuant TL (Amersham Biosciences) and represented as the percentage ratio of S100 to R100 signal intensities

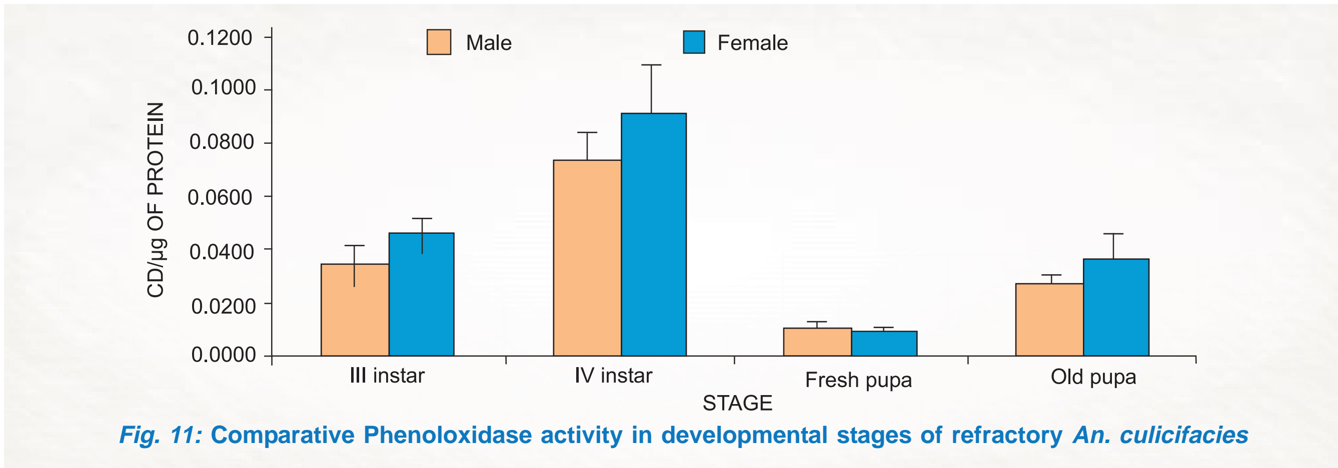


Fig. 11: Comparative Phenoloxidase activity in developmental stages of refractory *An. culicifacies*

pupae it was insignificantly higher than in the corresponding male (Fig. 11).

Zymogram showed two clear bands of MPO and DPO in both the sexes of III instar larvae. Similarly, IV instar male larvae also had two clear bands, while IV instar female larvae showed only one comparatively darker and broader band (Fig. 12). Fresh male and female pupae showed single band while older pupae had two bands (Fig. 13). Activity of MPO is found slightly higher than the DPO on SDS-PAGE.

1.5.2 Biochemical and molecular characterisation of Nitric oxide synthase in *An. culicifacies*: relevance for refractory mechanism

Earlier we reported the specific activities of *An. culicifacies* Nitric Oxide Synthase (AcNOS) in lysates of non-blood fed, uninfected or *P. falciparum*-infected mosquitoes at 6 and 9 days post-blood meal activity with or without NOS inhibitor L-NAME and amplified the NOS gene in *An. culicifacies* species A and species B and established NOS as an informative marker.

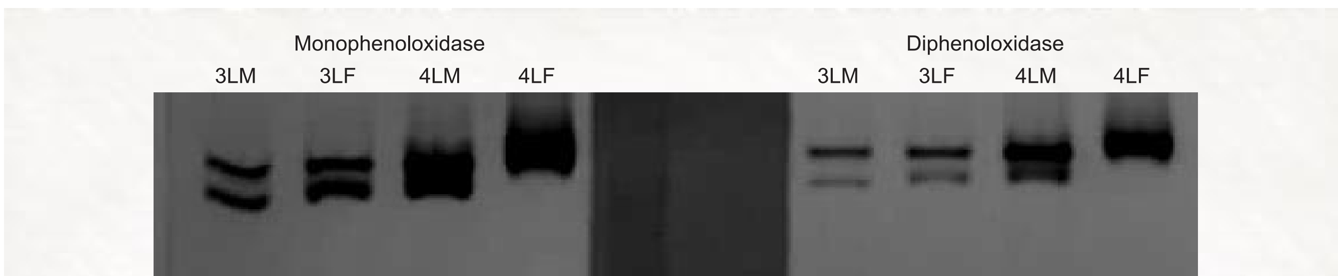


Fig. 12: MPO & DPO activities in IV and III instar larvae on SDS-PAGE

Fig. 13: MPO & DPO activities in freshly emerged and older pupae on SDS-PAGE (3LM: III instar male larvae; 4LM: IV instar male larvae; 3LF: III instar female larvae; 4LF: IV instar female larvae; FPM: Fresh male pupae; OPM: Older male pupae; FPF: Fresh female pupae; OPF: Older female pupae)

In continuation with our earlier studies, we have identified and characterised expression of AcNOS, which is highly homologous to characterised NOS genes and was detected in the midgut soon after invasion of the midgut by *P. vivax* at the beginning of blood feeding. Increased levels of mRNAs (encoding iNOS) were observed 9–14 days after ingestion of an infected blood meal using RT-PCR analyses (Fig. 14). *An. culicifacies* NOS (AcNOS) expression was studied by using semi-quantitative RT-PCR. Total RNA was isolated from 30–40 each non-blood fed, blood fed uninfected, and *P. vivax*-infected mosquitoes at 6, 9, 14, and 18 days pBM. A strong induction occurred in infected mosquitoes at 7 days and 9–14 days. Semiquantitative RT-PCR experiments established that AcNOS is parasite inducible as shown by significant increase in transcript levels (9–14 days) after feeding parasite-infected blood to mosquitoes (Fig. 14).

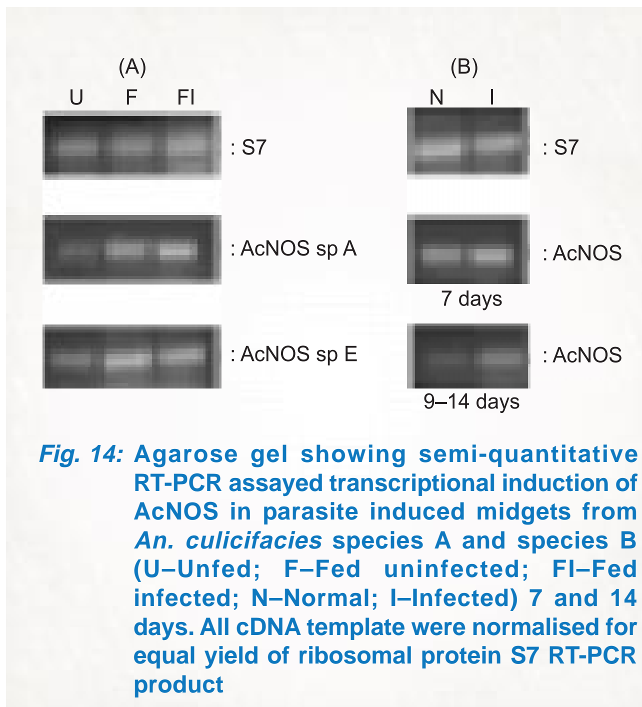


Fig. 14: **Agarose gel showing semi-quantitative RT-PCR assayed transcriptional induction of AcNOS in parasite induced midgets from *An. culicifacies* species A and species B (U–Unfed; F–Fed uninfected; FI–Fed infected; N–Normal; I–Infected) 7 and 14 days. All cDNA template were normalised for equal yield of ribosomal protein S7 RT-PCR product**

Total RNA from midguts of blood fed uninfected and *P. vivax* blood fed infected was assayed by using semiquantitative RT-PCR for AcNOS at 1, 7, 9, 14 days post-blood meal. Transcript abundance was normalised against S7 protein gene to correct for sample variations in PCR template (Fig. 15).

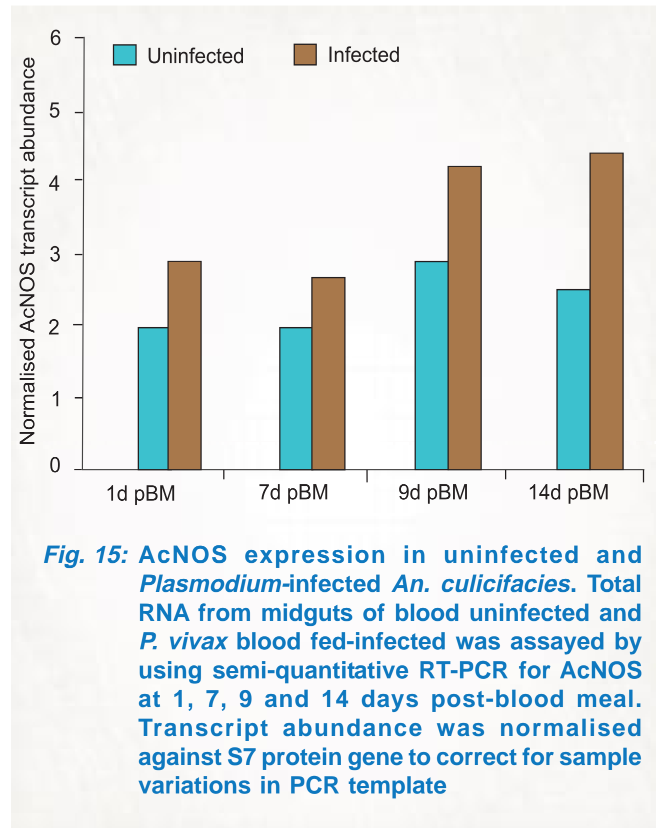


Fig. 15: **AcNOS expression in uninfected and *Plasmodium*-infected *An. culicifacies*. Total RNA from midguts of blood uninfected and *P. vivax* blood fed-infected was assayed by using semi-quantitative RT-PCR for AcNOS at 1, 7, 9 and 14 days post-blood meal. Transcript abundance was normalised against S7 protein gene to correct for sample variations in PCR template**

Analysis of the molecular interactions between *An. culicifacies* midgut epithelial cells and *P. vivax* parasites revealed that iNOS may be used as an additional effector gene to block the development of the malarial parasite in mosquitoes. Biological significance of enhanced gene expression and enhanced enzyme activity is unknown. However, correlation with sporozoite release time suggests that mosquito NO may interfere with this phase of parasite development and may lead to a mechanism of refractoriness. Such responses may be important for the vectorial capacity of the mosquito, understanding of parasite-vector interactions and elucidated mechanism of refractoriness.