Sublethal and transgenerational effects of alphamethrin on life history traits of *Anopheles stephensi* (Diptera: Culicidae), a malaria mosquito

T.P.N. Hariprasad,¹ N.J. Shetty²

Abstract—*Anopheles stephensi* Liston (Diptera: Culicidae), an urban malaria vector in India, is controlled by insecticides. Sublethal concentrations can be effective in reducing mosquito populations and disease transmission by reducing its reproductive fitness. In this study, sublethal effects of alphamethrin, a synthetic pyrethroid, were assessed on selected fitness parameters. Late third instars of the Goraguntepalya strain, Bangalore, were exposed to three sublethal doses, $LC_{10} = 0.00006$, $LC_{30} = 0.0004$, and $LC_{50} = 0.0014$ mg/L and their effects on fecundity, egg hatchability, sex ratio, and longevity in parental, F_1 and F_2 generations. The morphology of gonads, pattern of pupation, and adult emergence were also assessed. We found a significant reduction in fecundity and hatchability among the sublethal concentrations as well as across generations. Survival analysis showed significant reduction in lifespan of exposed groups. Delay in pupation, eclosion, and no distortion in sex ratio was observed. The results suggest that sublethal concentrations of alphamethrin may have negative effects on exposed individuals and subsequent generations.

Introduction

The ability of insecticides to eliminate huge numbers of mosquitoes (Diptera: Culicidae) interrupts disease transmission, but on the other hand, imposes immense selection for resistance (Shetty 1997; Glunt *et al.* 2011; Insecticide Resistance Action Committee 2011; Centers for Disease Control and Prevention 2015; Liu 2015; World Health Organization 2015). Malaria control now is largely dependent on insecticides belonging to synthetic pyrethroids (World Health Organization 2013) but their efficacy is now under threat due to the development of resistance in mosquito vectors worldwide (Ranson *et al.* 2009). Resistant mosquitoes can spread into different geographic regions and reduce the effectiveness of mosquito control programmes (Zaim and Guillet 2002; Ranson *et al.* 2011).

Treatment of a target species with an insecticide may be uneven as the insecticide deposit is a result

of complicated and dynamic interactions between biotic and abiotic factors, such as vegetation, wind speed and direction, rainfall, *etc.*, and therefore the insects may receive sublethal concentrations of the toxin and the insecticide performance may be often greatly reduced (Rust 1995; Ali *et al.* 2006). Sublethal effects are the biological, physiological, and behavioural changes of surviving insects following contact with a sublethal amount of the insecticide (Lee 2000; Desneux *et al.* 2007; Biondi *et al.* 2012a). The sublethal effects are particular to each insecticide, which depends on the mode of action, life stage of the insect exposed, duration of exposure, concentration, and environmental factors such as temperature and food (Perez *et al.* 2007).

The older mosquitoes are more susceptible to insecticides and the risk of mortality increases after blood feeding (Rowland and Hemingway 1987), therefore low concentrations of the insecticide could remove the older females

Received 29 September 2015. Accepted 4 August 2016. First published online 7 February 2017.

T.P.N. Hariprasad, Centre for Applied Genetics, Jnana Bharathi, Bangalore University, Bangalore 560 056, India

N.J. Shetty,² Professor Emeritus, Centre for Applied Genetics, Jnana Bharathi, Bangalore University, Bangalore 560 056, India

¹Present Address: Yenepoya Research Centre, Yenepoya University, Deralakatte, Mangalore 575018, India.

²Corresponding author (e-mail: shetty_nj@yahoo.co.in).

Subject editor: Kateryn Rochon

doi:10.4039/tce.2016.57

substantially (Glunt *et al.* 2011). In addition, females harbouring parasites are less tolerant to insecticide exposure as the parasites impose an additional metabolic stress on them (Molina-Cruz *et al.* 2008). Apparent reduction in the infectivity of insecticide treated *Anopheles stephensi* Liston (Diptera: Culicidae) with rodent malaria, *Plasmodium yoelii yoelii* Landau, Michel, and Adam (Protista: Plasmodiidae) was described (Carle *et al.* 1986) suggesting that the reduction could be due to the direct toxicity of the insecticide on the parasite.

Studies on sublethal effects of insecticides have been carried out to discover the negative and non-lethal impacts of insecticides on life history traits that might affect population dynamics (Stark and Banks 2003). Accurate assessment of sublethal effects is crucial in controlling insect pest populations and understanding their selectivity towards non-target organisms (Desneux *et al.* 2007; Tan *et al.* 2012; Biondi *et al.* 2012b).

Anopheles stephensi is an urban malaria vector in the Indian subcontinent and accounts for about 15% of the annual malaria incidence (Shetty 2002). In southeastern Asia, India has the highest burden of malaria (World Health Organization 2012). Under the auspices of World Health Organization Pesticide Evaluation Scheme, alphamethrin (= alphacypermethrin) is recommended for malaria prevention and control (World Health Organization 2009).

The purpose of this paper is to describe the sublethal effects of alphamethrin on life history traits like fecundity, hatchability, sex ratio, and longevity for the exposed parental generation, as well as subsequent generations without insecticide exposure (F_1 and F_2). This paper also describes the sublethal effects of alphamethrin on genital morphology, rhythms of pupation, and adult emergence (eclosion) in *A. stephensi*.

Material and methods

Anopheles stephensi strain and maintenance

Anopheles stephensi strain of Goraguntepalya (GGP), Bangalore, India was used in the present investigation. The said strain was collected as larvae from cement tank in a construction area. Larvae were reared in white enamel pans in the insectary. The pupae formed from these larvae were transferred into wide-mouth bottles and the

emerging adults were released into cages made of iron frames covered with nylon mosquito net. The adults were fed with sucrose solution (10%) soaked in sterilised cotton. Upon maturation the females were provided blood meal from restrained mice. Plastic vials (7.62 cm diameter) lined by filter paper and filled with water were placed inside the cage for oviposition. The laid eggs were kept for 72 hours to ensure complete hatching. The hatched larvae were transferred to enamel pans containing filtered tap water and were fed with powdered yeast tablets throughout the larval period. These stocks were maintained at a temperature of $25 \pm 1^\circ\text{C}$ with relative humidity of $75 \pm 5\%$ and 10 hours of photoperiod throughout the course of investigations (Shetty 1983). The said colony was maintained for 10 generations in the insectary without any insecticide exposure.

Insecticide and larval bioassay

Alphamethrin (96.7% technical concentration), a synthetic pyrethroid (supplied by Tata Rallies India Limited, Mumbai, India), acts as nerve poison with very low doses and is effective against a range of pests in agriculture, public health, and animal husbandry. Alphamethrin (Type II pyrethroid) is a racemic mixture of (*S*)- α -cyano-3-phenoxybenzyl-(1*R*,3*R*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate and (*R*)- α -cyano-3-phenoxybenzyl-(1*S*,3*S*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate having the molecular formula, $\text{C}_{22}\text{H}_{19}\text{Cl}_2\text{NO}_3$. Type II pyrethroids cause prolonged opening of sodium channel resulting in the influx of sodium ions into the cell. This causes membrane depolarisation accompanied by suppression of the action potential (Gammon *et al.* 1981; Lund and Narahashi, 1981).

Larval bioassay (in four replicates) was carried out for series of concentrations ranging from 0.00001 to 0.1 mg/L of alphamethrin (World Health Organization 2005). Mortality was recorded 24 hours post exposure and the mortality data was subjected to probit analysis. LC_{10} , LC_{30} , and LC_{50} were calculated following regression line method (Finney 1971). LC_{10} , LC_{30} , and LC_{50} were found to be 0.00006, 0.0004, and 0.0014 mg/L, respectively.

Sublethal and test concentrations of alphamethrin

Stock concentration of insecticide was prepared by dissolving alphamethrin in denatured alcohol

(98 mL of absolute alcohol + 2 mL ethyl methyl ketone). From the known stock concentration, the three sublethal concentrations (LC₁₀, LC₃₀, and LC₅₀) were prepared by serial dilution using denatured alcohol.

Test concentrations were prepared by adding 1 mL of the sublethal concentration in 249 mL of water. Triplicates of 25 late third instars were introduced to the test solutions. Parallel controls were setup by adding 25 larvae and 1 mL denatured alcohol in 249 mL water. Mortality was observed 24 hours after exposure (World Health Organization 2005). The survivors were washed with tap water and reared in separate enamel pans and fed on powdered yeast tablets. The adults emerged from the exposed larvae was the parental generation. F₁ and F₂ generations were subsequently obtained by selfing males and females of respective generations. Parental, F₁ and F₂ generations were obtained for each sublethal concentration. Observations on changes in life history parameters were done as described by Aguilera *et al.* (1995) and Zin and Shetty (2008).

Fecundity

Each blood fed female from the above treatments was separated and introduced into a 35-mL vial lined with filter paper and 10 mL of water for oviposition. The filter paper on which the eggs were laid was collected and eggs were counted under the dissection microscope at 20× magnification.

Hatchability

The eggs of each female were allowed to hatch and the larvae hatched were counted. The per cent hatchability for each iso-female was calculated using the formula:

$$\% \text{ Hatchability} = \frac{\text{Number of larvae}}{\text{Number of eggs}} \times 100$$

Sex ratio

The larvae of each female were reared as a separate iso-family. Precautions were taken to avoid selective mortality as a source of bias in collection of data for sex ratio distortion. If mortality of larvae exceeded 10%, the entire family was discarded. The number of freshly emerged males and females were scored at each generation for each female and the male to female ratio was recorded.

Longevity

The adults emerged from larvae exposed to sublethal concentrations were reared as separate stock. The number of days they survived from the day of emergence was taken as longevity of the adult flies and was recorded.

Morphology of gonads

Five-day-old adult male and female mosquitoes, whose larval stage was treated for LC₁₀, LC₃₀, and LC₅₀, were used for studying morphological variations in the gonads. Males and females were separated into two test tubes and were incapacitated by tapping the lower end of the test tube against the palm. Once disabled, each adult was placed in a drop of insect saline on a slide and its reproductive system was removed using needles under a dissecting microscope (×40). In the males, their penultimate abdominal segment was pulled out using a needle to get the intact reproductive system including testes, accessory glands, and ejaculatory duct. The female reproductive system was dissected by placing one needle on the anterior part of the abdomen and by pulling out the last two abdominal segments using another needle. The reproductive systems were separated from debris on each slide and examined for structural abnormalities under the dissection microscope. A total of 50 males and females for each concentration were dissected. Control (untreated) males and females were also dissected for comparison.

Rhythms of pupation and eclosion

The late third instars were exposed to the three sublethal concentrations of alphamethrin and their effects on pupation and eclosion were studied following the method of Nayar and Sauerman (1970). The patterns of larval development leading to pupation and pupal development leading to eclosion were recorded. Four batches of 25 larvae, recovered after being exposed to sublethal concentration for 24 hours were reared in separate trays. The time taken for the recovered larvae to pupate (pupation) was recorded. Pupae thus formed were collected in a wide-mouth bottle. The time taken for the pupae to emerge (eclosion) was recorded. The number of pupae formed and adults emerged were recorded every two hours. Parallel to this, controls were set up with 25 larvae each. Pupation and eclosion patterns of all the

exposed groups were compared with that of the control.

Statistical analysis

Mortality data recorded after 24 hours of treatment of the larvae were subjected to probit analysis (Finney 1971) for calculating LC₁₀, LC₃₀, and LC₅₀. Data from fecundity hatchability and pupation were analysed by analysis of variance (ANOVA) with post-hoc Tukey’s honest significant difference test, sex ratio was analysed by ANOVA with Tukey’s test as well as by *t*-test, longevity data was analysed by *t*-test and Kaplan–Meier survival analysis with Wilcoxon χ^2 test, and eclosion data was analysed by Mann–Whitney test using SPSS software package version 15.0 for Windows (SPSS, Chicago, Illinois, United States of America). For all the above tests, probability level, $P < 0.05$ was determined as significant. All data were checked for homogeneity of variance and normality (transformation).

Results

Sublethal concentrations

The three sublethal concentrations (LC₁₀, LC₃₀, and LC₅₀) with 95% fiducial limits obtained for

the strain GGP from the regression equation ($y = 1.96 + 0.959x$) were LC₁₀ : 0.00006 (0.000033–0.000081), LC₃₀: 0.0004 (0.00019–0.00060), and LC₅₀ : 0.0014 (0.0011–0.0016).

Direct and transgenerational effects of sublethal concentrations of alphasmethrin on fecundity, egg hatchability, sex ratio, and morphology of gonads

Fecundity. Reduction in the fecundity was observed in the exposed groups. A gradual reduction was also found with increase in concentrations (Table 1). The sublethal concentrations significantly reduced fecundity among concentrations in parental ($F(3,56) = 21.8, P < 0.0001$), F₁ ($F(3,56) = 49.3, P < 0.0001$), and F₂ ($F(3,56) = 97.332, P < 0.0001$) generations.

Tukey’s post-hoc test showed that fecundity of the parental generation was reduced at LC₁₀ ($P = 0.05$), LC₃₀ ($P < 0.0001$), and LC₅₀ ($P < 0.0001$) when compared with its control. Fecundity was also greatly reduced when compared between LC₁₀ and LC₅₀, LC₃₀, and LC₅₀ however the reduction was not significant between LC₁₀ and LC₃₀ ($P = 0.188$). A similar trend was observed in F₁ generation, where the fecundity between LC₁₀ and LC₃₀, LC₃₀ and LC₅₀ were not so different.

Table 1. Sublethal effects of alphasmethrin on fecundity and hatchability of *Anopheles stephensi*.

Strain: GGP (n = 15)					
Generation/ dosage	Fecundity		Hatchability		
	Eggs/female (Mean ± SE)	Ratio	Larvae/female (Mean ± SE)	Per cent hatchability	Ratio
Parental					
Control	126.13 ± 1.84	1.00	113.40 ± 1.99	88.90	1.00
LC ₁₀	115.33 ± 3.64	0.91	87.00 ± 2.74	73.35	0.83
LC ₃₀	106.93 ± 3.56	0.85	76.93 ± 3.11	71.94	0.81
LC ₅₀	93.88 ± 2.51	0.74	62.00 ± 2.09	66.05	0.74
F ₁					
Control	110.60 ± 1.95	1.00	98.00 ± 2.35	88.60	1.00
LC ₁₀	89.00 ± 2.16	0.80	69.67 ± 1.64	78.28	0.88
LC ₃₀	81.00 ± 2.26	0.73	58.00 ± 1.74	71.60	0.81
LC ₅₀	73.60 ± 2.80	0.67	50.60 ± 2.48	68.75	0.78
F ₂					
Control	116.73 ± 1.66	1.00	99.86 ± 2.76	85.55	1.00
LC ₁₀	83.33 ± 2.23	0.71	62.33 ± 2.30	74.80	0.87
LC ₃₀	68.53 ± 2.20	0.59	46.00 ± 1.71	67.12	0.78
LC ₅₀	61.33 ± 3.20	0.53	33.88 ± 2.04	55.00	0.64

Notes: n = number of females. GGP, Goraguntepalaya.

Fig. 1. Mean ($\pm$ standard error) fecundity (A, B), hatchability (C, D), and sex ratio (E, F) in *Anopheles stephensi* exposed to sublethal concentrations of alphamethrin. A, C, E: among concentrations; B, D, F: across generations. C: control; LC₁₀, LC₃₀, LC₅₀: sublethal concentrations. P, F₁, F₂: parental and filial generations. Mean bars with identical letters indicate non-significance.

Fecundity in F₂ generation was also reduced compared with control and among other groups, however the reduction was not so significant between LC₃₀ and LC₅₀ (Fig. 1A).

A significant reduction in fecundity was observed across generations (parental, F₁ and F₂)

at LC₁₀ ($F(2,42) = 35.62$, $P < 0.0001$), LC₃₀ ($F(2,42) = 52.164$, $P < 0.0001$), and LC₅₀ ($F(2,42) = 34.296$, $P < 0.0001$). Tukey's post-hoc test showed that fecundity at LC₁₀ was reduced in parental generation when compared with F₁ and F₂ ($P < 0.0001$). However there was no significant

reduction between F_1 and F_2 ($P = 0.350$). Fecundity at LC_{30} and LC_{50} was found to be reduced when compared among all groups ($P = 0.01$) (Fig. 1B).

Egg hatchability. Reduction in the egg hatchability was observed in the exposed groups. Similar to fecundity, gradual reduction was found with increase in concentrations (Table 1). The sublethal concentrations significantly reduced egg hatchability among concentrations in parental ($F(3,56) = 73.419$, $P < 0.0001$), F_1 ($F(3,56) = 99.75$, $P < 0.0001$) and F_2 ($F(3,56) = 164.82$, $P < 0.0001$) generations.

Tukey's post-hoc test showed that egg hatchability of parental generation was reduced at LC_{10} , LC_{30} , and LC_{50} ($P < 0.001$) when compared with its control. Egg hatchability was also greatly reduced when compared between all groups, *i.e.*, LC_{10} and LC_{30} , LC_{10} and LC_{50} , as well as LC_{30} and LC_{50} ($P < 0.05$). A similar trend was observed in F_1 where hatchability was significantly reduced among all the groups ($P = 0.001$) except between LC_{30} and LC_{50} ($P > 0.05$). Egg hatchability in the F_2 generation was reduced compared with control, and among all the groups ($P = 0.01$) (Fig. 1C).

The significant reduction in egg hatchability was observed across parental, F_1 and F_2 generations,

at LC_{10} ($F(2,42) = 31.084$, $P < 0.0001$), LC_{30} ($F(2,42) = 46.692$, $P < 0.0001$), and LC_{50} ($F(2,42) = 40.88$, $P < 0.0001$).

Tukey's post-hoc test showed that egg hatchability at LC_{10} was reduced in parental generation when compared with F_1 and F_2 ($P < 0.001$), however there was no significant difference in hatchability between F_1 and F_2 ($P > 0.05$). Egg hatchability showed reduction at LC_{30} and LC_{50} among all the groups ($P < 0.01$) (Fig. 1D).

Sex ratio. The sex ratio was tilted towards the males, *i.e.*, the number of males were more than the females in treated and as well as in control (Table 2). Sublethal concentrations reduced the male to female ratio among concentrations in parental generation ($F(3,56) = 4.828$, $P = 0.005$), however there were no differences in F_1 ($F(3,56) = 2.265$, $P = 0.091$) and F_2 ($F(3,56) = 0.113$, $P = 0.952$) generations.

In the parental generation the decrease in the sex ratio was detected at LC_{10} ($P = 0.006$) when compared with control. The difference was also significant between LC_{10} and LC_{50} ($P = 0.037$). There were no differences among the other groups in this generation. There were also no differences in sex ratios in F_1 or F_2 (Fig. 1E).

Table 2. Sublethal effects of alphasmethrin on sex ratio and longevity of *Anopheles stephensi*.

Strain: GGP (<i>n</i> = 15)	Sex ratio			Longevity (days) (mean $\pm$ SE)	
	Number of males	Number of females	Male: female	Males	Females
Parental					
Control	843	740	1: 0.88	22.53 $\pm$ 0.45	35.00 $\pm$ 0.91
LC_{10}	703	495	1: 0.70	19.50 $\pm$ 0.40	26.53 $\pm$ 0.82
LC_{30}	619	471	1: 0.76	21.00 $\pm$ 1.00	23.53 $\pm$ 1.23
LC_{50}	480	405	1: 0.84	22.00 $\pm$ 0.91	26.00 $\pm$ 1.15
F_1					
Control	646	585	1: 0.91	21.20 $\pm$ 0.58	33.40 $\pm$ 0.42
LC_{10}	543	444	1: 0.82	18.93 $\pm$ 0.53	29.20 $\pm$ 0.61
LC_{30}	447	377	1: 0.84	20.70 $\pm$ 0.38	27.00 $\pm$ 0.37
LC_{50}	376	345	1: 0.92	19.86 $\pm$ 0.42	27.66 $\pm$ 0.12
F_2					
Control	739	632	1: 0.86	21.40 $\pm$ 0.74	34.80 $\pm$ 0.38
LC_{10}	470	410	1: 0.87	19.66 $\pm$ 0.73	30.00 $\pm$ 0.63
LC_{30}	354	294	1: 0.83	19.20 $\pm$ 0.47	28.66 $\pm$ 0.50
LC_{50}	261	221	1: 0.85	17.46 $\pm$ 0.70	29.40 $\pm$ 0.53

Notes: *n* = number of females. GGP, Goraguntepalaya.

Significant reduction in sex ratio was observed across parental, F_1 and F_2 generations at LC_{10} ($F(2,42) = 6.470$, $P = 0.004$), however there were no differences at LC_{30} ($F(2,42) = 1.283$, $P = 0.288$) and LC_{50} ($F(2,42) = 0.626$, $P = 0.539$).

Reduction in sex ratio at LC_{10} was detected only between parental and F_2 generations ($P = 0.003$). There were no differences between other groups at LC_{10} and also no differences among all the groups at LC_{30} and LC_{50} (Fig. 1F).

Longevity. The life span of females was longer than males in control and sublethal concentrations. Overall reduction in lifespan of exposed groups was recorded in comparison with the control (Table 2). Kaplan–Meier survival analysis showed significant reduction in male longevity in parental ($\chi^2 = 9.85$, $df = 3$, $P = 0.02$), F_1 ($\chi^2 = 12.801$, $df = 3$, $P = 0.005$), and F_2 ($\chi^2 = 16.554$, $df = 3$, $P = 0.001$) generations. Similarly female longevity was also significantly reduced in parental ($\chi^2 = 28.409$, $df = 3$, $P < 0.0001$), F_1 ($\chi^2 = 38.232$, $df = 3$, $P < 0.0001$), and F_2 ($\chi^2 = 29.243$, $df = 3$, $P < 0.0001$) generations (Fig. 2).

Post-hoc test showed that male longevity in parental generation was reduced at LC_{10} ($P = 0.030$) when compared with control, however there were no differences among other groups. A similar trend was observed in the F_1 generation where longevity was reduced at LC_{10} ($P = 0.010$) in comparison with control. Longevity in the F_2 generation was only reduced at LC_{50} ($P = 0.001$) when compared with controls (Fig. 3A).

Male longevity showed no differences among generations at LC_{10} . Longevity was reduced at LC_{50} only between F_1 and F_2 ($P = 0.023$). Longevity at LC_{50} was reduced among all the groups ($P < 0.05$) (Fig. 3B).

Female longevity in parental generation was reduced at LC_{10} , LC_{30} , and LC_{50} ($P < 0.0001$) when compared with control, however no differences were found between groups. A similar trend was observed in F_1 as well as F_2 generations ($P < 0.0001$) (Fig. 3C). Female longevity at LC_{10} was reduced between parental and F_1 ($P = 0.015$) and F_2 ($P = 0.002$) generations. Longevity at LC_{30} was reduced among all the generations. Longevity at LC_{50} was reduced between parental

and F_2 ($P = 0.014$) as well as F_1 and F_2 ($P = 0.006$) (Fig. 3D).

Independent *t*-tests showed that the longevity of the females in the parental generation was longer when compared with that of the males in LC_{10} ($P < 0.0001$) and LC_{50} ($P = 0.011$). Longevity of females was also longer among all the groups in F_1 and F_2 generations ($P < 0.0001$) (Fig. 3E).

Morphology of gonads. No morphological abnormalities were observed on sublethal exposures of alphamethrin in reproductive systems of males and females. No structural abnormalities were observed in testicular lobules, vas deferens, accessory glands, or ovaries among exposed groups as compared with control.

Direct effects of sublethal concentrations of alphamethrin on rhythms of pupation and rhythms of eclosion

Rhythms of pupation. Pupation in control larvae began at 48 hours post recovery from sublethal exposure. Mean pupation time was found to be 59 ± 1.91 (mean $\pm$ standard error) hours. Maximum pupation peak occurred at 52–58 hours and was completed by 70 hours. Similarly, LC_{10} exposed larvae began pupation at 60 hours post recovery. The mean pupation was found to be 73 ± 2.08 hours. Maximum peak occurred at 62–66 hours and continued as smaller peaks up to 86 hours. Among LC_{30} exposed larvae pupation also began at 60 hours post recovery. Mean pupation time was found to be 77 ± 2.38 hours. Maximum pupation peak occurred at 62–66 hours and continued as smaller peaks up to at 94 hours. Larvae pupated were fewer than control and LC_{10} . Similarly LC_{50} exposed larvae also began pupation at 60 hours but the number of larvae pupated were fewer than control and other exposed groups. Mean pupation time was 80 ± 2.58 hours. Maximum peak occurred at 62 hours and continued as very small peaks up to 98 hours. Pupation was found to be delayed by 14, 18, and 21 hours post recovery for LC_{10} , LC_{30} , and LC_{50} , respectively (Fig. 4). The results indicate toxicological effects of sublethal concentrations on the rhythms of pupation.

Sublethal concentrations significantly delayed pupation among concentrations ($F(3,53) = 12.019$, $P < 0.0001$). Tukey's post-hoc comparisons showed that the delay in pupation was

Fig. 2. Kaplan–Meier survival graphs for sublethal exposure of alphamethrin on males and females of *Anopheles stephensi* in parental, F₁ and F₂ generations.

significant at LC₁₀ ($P = 0.005$), LC₃₀ ($P < 0.0001$), as well as LC₅₀ ($P < 0.0001$) when compared with control. However there were no differences among the other groups.

Rhythms of eclosion. The adults emerged from larvae exposed to sublethal concentrations were

released into their respective cages and scored for sex. The pupal duration was prolonged and eclosion delayed in exposed groups. It was observed that the males emerged much earlier than females among the treated groups and also in the control. In the control group, maximum eclosion occurred at 28–32 hours (males) and

Fig. 3. Mean ($\pm$ standard error) male (A, B), female (C, D), and comparative (E) longevity in *Anopheles stephensi* exposed to sublethal concentrations of alphamethrin. A, C, E: among concentrations; B, D: across generations. C: control; LC₁₀, LC₃₀, LC₅₀: sublethal concentrations. P, F₁, F₂: parental and filial generations. Mean bars with identical letters indicate non-significance.

68–72 hours (females) post pupation. The mean ($\pm$ standard error) eclosion times were 40 ± 3.26 and 41 ± 3.21 hours, respectively. The mean eclosion time of LC₁₀ exposed males was also 42 ± 3.16 and females was 48 ± 3.05 hours. Male emergence showed two major peaks at

34–38 hours and 44–48 hours while the females showed two major peaks at 54–58 hours and 68–72 hours indicating delay in eclosion. LC₃₀ exposed larvae showed a maximum male emergence at 54–58 hours and female emergence at 68–72 hours. The mean emergence times were

Fig. 4. Rhythms of pupation on exposure to sublethal concentrations of alphamethrin in *Anopheles stephensi*.

46 ± 3.16 and 55 ± 2.64 hours, respectively. Similarly LC_{50} exposed larvae showed two male emergence peaks at 46–48 and 54–58 hours and two female emergence peaks at 68–72 and 74–78 hours. The mean emergence times were 58 ± 2.58 and 61 ± 2.38 hours, respectively. The delay of eclosion was clearly evident when the larvae were treated to the sublethal concentrations (Fig. 5).

Fig. 5. Rhythms of adult eclosion on exposure to sublethal concentrations of alphamethrin in *Anopheles stephensi*.

Mann–Whitney comparisons showed delay in male emergence at LC_{50} ($P = 0.001$) while no significant delay at LC_{10} and LC_{30} when compared with control. Also the delay was not significant between LC_{10} and LC_{30} (Fig. 6A). Comparison showed delay in female emergence at LC_{30} ($P = 0.006$) and LC_{50} ($P = 0.0001$) when compared with control and no significant delay at LC_{10} ($P = 0.139$) (Fig. 6A). Mann–Whitney comparisons showed no significant delay in male to female eclosion among all the groups (Fig. 6B).

Fig. 6. Mean ($\pm$ standard error) males and females (A), comparative (B) eclosion in *Anopheles stephensi* exposed to sublethal concentrations of alphamethrin. C: control; LC₁₀, LC₃₀, LC₅₀: sublethal concentrations. Mean bars with identical letters indicate non-significance.

Discussion

As insecticides are one of the reliable control methods, refining the insecticidal control measures could provide effective malaria control while considerably reducing selection for resistance (Gourley *et al.* 2011). The life history traits studied were reduced significantly in the exposed groups, which indicates that alphamethrin caused reproductive disadvantages in the exposed groups.

In this study, fecundity and egg hatchability were significantly reduced. Fecundity could be reduced due to physiological and behavioural effects caused by insecticides (Desneux *et al.* 2007) and also could be due to the penetration of the chemical into the egg (Broadbent and Pree 1984). Insecticides can disrupt the metabolic process in insects, which affect behaviour and physiology that do not lead to direct mortality. Therefore, insecticides decrease the production of offspring because of behavioural modifications in mate location, courtship, and oviposition, or due to physiological effects on egg fertilisation, oogenesis, ovulation, spermatogenesis, sperm motility, and overall affecting population structure (Haynes 1988; Ali *et al.* 2006).

The effects of insecticides on various reproductive parameters such as fecundity, hatchability, and sex ratio were reported in *A. stephensi* for neem and bifenthrin (Zin and Shetty 2008); fenitrothion, cypermethrin, and deltamethrin (Priyalakshmi *et al.* 1999); and propoxur

and temephos (Sanil and Shetty 2012). Pyriproxyfen (2%) showed no effects on fecundity but the fertility of eggs showed significant reduction in *A. stephensi* (Aiku *et al.* 2006). Decrease in fecundity was also observed in *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) to alphamethrin and malathion (Minn and Shetty 2008).

No sex ratio distortion was observed in this study suggesting that alphamethrin may be indiscriminative towards the developing larvae. Cytological observations indicated that sex ratio bias towards male in *Aedes* Meigen is associated with preferential breakage of the X chromosome during male meiosis (Newton *et al.* 1976). Molecular mechanisms for sex ratio distortion was attributed to highly specific homing endonuclease I-PpoI7, which cuts a conserved sequence on the mosquito's (*Anopheles gambiae* Giles) X chromosome and experiments indicated that the majority of developmentally arrested embryos in *A. gambiae* had been fertilised by Y-bearing spermatozoa (Windbichler *et al.* 2007, 2008).

Longevity is an essential component of reproductive fitness, which can potentially increase the magnitude of disease transmission. Longevity of insects treated with sublethal concentrations of insecticides may increase, decrease, or remain unchanged (Lee 2000). In the present study, the longevity of males and females was decreased in the exposed groups as compared with control. Longevity of females is usually longer, as also in this study. The reduction in male longevity can

reduce the number of matings and reduction in female longevity can reduce the number of ovipositions, which would also lead to reduced fecundity. Importantly, the female mosquito might die before the end of the incubation period of the parasite.

A sharp decrease in life span was observed in adults whose larval stages were exposed to sublethal concentrations of hexaflumuron in *Culex quinquefasciatus* Say, *A. stephensi*, and *A. aegypti* (Vasuki 1992). Sublethal exposures of propoxur and temephos were found to prolong the lifespan of *A. stephensi* (Sanil and Shetty 2012) and temephos treated larval stages in *A. aegypti* (Reyes-Villanueva *et al.* 1990).

This study showed irregular rhythms of pupation and eclosion of larvae exposed to sublethal concentrations of alphamethrin as compared with control. The pupation and eclosion rhythms were found to be disturbed on exposure to sublethal concentrations of temephos and propoxur in *A. stephensi* (Sanil and Shetty 2012). Sublethal concentrations of malathion, methoprene, and resmethrin resulted in significantly lengthened time to pupation. The time needed for adults of insecticide-exposed mosquito populations to emerge was greater than controls for the malathion, methoprene, and resmethrin (Robert and Olson 1989). Pyriproxyfen (Sumilar®0.5G) inhibited over 80% of the total adult emergence in *A. gambiae* (Mbare *et al.* 2013).

The male and female gonads of treated groups were dissected out and observed for structural abnormalities. No structural abnormalities were observed. On the contrary, temephos and propoxur treated males showed atrophied and rudimentary testes, reduced accessory glands and short, disintegrated, thin vas deferens, *etc.* (Sanil and Shetty 2012).

Even though there are no structural abnormalities, the sublethal effects show reduction in fecundity, which may be due to neurotoxic action of alphamethrin that would disrupt neurohormonal balance. Insecticides that act on the central nervous system disrupt normal reproductive physiology as reproduction is neurohormone regulated, neurohormonal imbalance from insecticide poisonings may affect normal function (Maddrell and Reynolds 1972; Robert and Olson 1989; Reyes-Villanueva *et al.* 1990; Lee 2000).

Knowledge of these sublethal effects of insecticides could be important in the decision-making

process in integrated pest management programmes (Robert and Olson 1989). Direct sublethal effects not only negatively affect the various life traits but also can show transgenerational effects. Such effects drastically impact key demographic parameters (Guo *et al.* 2013).

Sublethal concentrations can play a vital role in control of insect vectors of disease. Reducing the vector population by altering its reproductive fitness would be effective without imparting a strong selection pressure for resistance. In the present study sublethal effects of alphamethrin showed reduced fecundity, hatchability, longevity, and delay in pupation and eclosion in the exposed generation and also subsequent generations in *A. stephensi*.

Acknowledgements

This work was supported by financial assistance from University Grants Commission (UGC) and Department of Science and Technology (DST), New Delhi to Professor N. J. Shetty.

References

- Aguilera, L., Marquetti, M.C., Navarro, A., and Bisset, J. 1995. Effects of three organophosphorus insecticides in the reproductive potential of *Culex quinquefasciatus*. *Memórias do Instituto Oswaldo Cruz*, **90**: 411–413.
- Aiku, A.O., Yates, A., and Rowland, M. 2006. Laboratory evaluation of pyriproxyfen treated bednets on mosquito fertility and fecundity. A preliminary study. *West African Journal of Medicine*, **25**: 22–26.
- Ali, A., Xue, R.D., and Barnard, D.R. 2006. Effects of sublethal exposure to boric acid sugar bait on adult survival, host-seeking, bloodfeeding behavior, and reproduction of *Stegomyia albopicta*. *Journal of the American Mosquito Control Association*, **22**: 464–468.
- Biondi, A., Desneux, N., Siscaro, G., and Zappalà, L. 2012a. Using organic-certified rather than synthetic pesticides may not be safer for biological control agents: selectivity and side effects of 14 pesticides on the predator *Orius laevigatus*. *Chemosphere*, **87**: 803–812.
- Biondi, A., Mommaerts, V., Smagghe, G., Viñuela, E., Zappalà, L., and Desneux, N. 2012b. The non-target impact of spinosyns on beneficial arthropods. *Pest Management Science*, **68**: 1523–1536.
- Broadbent, A.B. and Pree, D.J. 1984. Effects of diflubenzuron and BAY SIR 8514 on the oriental fruit moth (Lepidoptera: Olethreutidae) and the oblique banded leafroller (Lepidoptera: Tortricidae). *Journal of Economic Entomology*, **77**: 194–197.

- Carle, P.R., Coz, J., Elissa, N., Gasquet, M., Sannier, C., Richard, A., and Timon-David, P. 1986. Activite antiplasmodique intravectorielle d'un pyrethrinoid: la deltamethrine. *Comptes Rendus de l'Académie des Sciences*, **330**: 565–568.
- Centers for Disease Control and Prevention. 2015. *Anopheles* mosquitoes. Available from <http://www.cdc.gov/malaria/about/biology/mosquitoes/> [accessed 26 December 2015].
- Desneux, N., Decourtaye, A., and Delpuech, J.M. 2007. The sublethal effects of pesticides on beneficial arthropods. *Annual Review of Entomology*, **52**: 81–106.
- Finney, D.J. 1971. Probit analysis. 3rd edition, Cambridge University Press, Cambridge, United Kingdom.
- Gammon, D.W., Brown, M.A., and Casida, J.E. 1981. Two classes of pyrethroid action in the cockroach. *Pesticide Biochemistry and Physiology*, **15**: 181–191.
- Glunt, K.D., Thomas, M.B., and Read, A.F. 2011. The effects of age, exposure history and malaria infection on the susceptibility of *Anopheles* mosquitoes to low concentrations of pyrethroid. *Public Library of Science One*, **6**: e24968, doi: 10.1371/journal.pone.0024968.
- Gourley, S.A., Liu, R., and Wu, J. 2011. Slowing the evolution of insecticide resistance in mosquitoes: a mathematical model. *Proceedings of the Royal Society of London A*, **467**: 2127–2148.
- Guo, L., Desneux, N., Sonoda, S., Liang, P., Han, P., and Gao, X. 2013. Sublethal and transgenerational effects of chlorantraniliprole on biological traits of the diamondback moth, *Plutella xylostella* L. *Crop Protection*, **48**: 29–34.
- Haynes, K.F. 1988. Sublethal effects of neurotoxic insecticides on insect behavior. *Annual Review of Entomology*, **33**: 149–168.
- Insecticide Resistance Action Committee. 2011. Prevention and management of insecticide resistance in vectors of public health importance [online]. Insecticide Resistance Action Committee. Available from http://www.irac-online.org/content/uploads/VM-layout-v2.6_LR.pdf [accessed 9 September 2016].
- Lee, C.Y. 2000. Sublethal effects of insecticides on longevity, fecundity and behaviour of insect pests: a review. *Journal of Bioscience*, **11**: 107–112.
- Liu, N. 2015. Insecticide resistance in mosquitoes: impact, mechanisms, and research directions. *Annual Review of Entomology*, **60**: 537–559.
- Lund, A.E. and Narahashi, T. 1981. Modification of sodium channel kinetics by the insecticide tetramethrin in crayfish giant axons. *Neurotoxicology*, **2**: 213–229.
- Maddrell, S.H.P. and Reynolds, S.E. 1972. Release of hormones in insects after poisoning with insecticides. *Nature*, **236**: 404–406.
- Mbare, O., Lindsay, S.W., and Fillinger, U. 2013. Dose-response tests and semi-field evaluation of lethal and sub-lethal effects of slow release pyriproxyfen granules (Sumilarv(R) 0.5G) for the control of the malaria vectors *Anopheles gambiae sensu lato* [online]. *Malaria Journal*, **12**: 94, doi: 1475-2875-12-94.
- Minn, Z.M. and Shetty, N.J. 2008. Toxicological effect of malathion and alphamethrin on reproductive potential in *Aedes aegypti*, a yellow fever mosquito. *Pestology*, **32**: 39–43.
- Molina-Cruz, A., DeJong, R.J., Charles, B., Gupta, L., Kumar, S., Jaramillo-Gutierrez, G., and Barillas-Mury, C. 2008. Reactive oxygen species modulate *Anopheles gambiae* immunity against bacteria and *Plasmodium*. *Journal of Biological Chemistry*, **283**: 3217–3223.
- Nayar, J.K. and Sauerman, D.M. 1970. A comparative study and development in Florida mosquito. I. Effects of environmental factors on ontogenetic timings, endogenous diurnal rhythm and synchrony of pupation and emergence. *Journal of Medical Entomology*, **7**: 163–174.
- Newton, M.E., Wood, R.J., and Southern, D.I. 1976. A cytogenetic analysis of meiotic drive in the mosquito, *Aedes aegypti* (L.). *Genetica*, **46**: 297–318.
- Perez, C.M., Marina, C.F., Bond, J.G., Rojas, J.C., Valle, J., and Williams, T. 2007. Spinosad, a naturally derived insecticide, for control of *Aedes aegypti* (Diptera: Culicidae): efficacy, persistence, and elicited oviposition response. *Journal of Medical Entomology*, **44**: 631–638.
- Priyalakshmi, B.L., Rajashree, B.H., Ghosh, C., and Shetty, N.J. 1999. Effect of fenitrothion, deltamethrin and cypermethrin on reproductive potential and longevity of life cycle in *Anopheles stephensi* Liston, a malaria mosquito. *Journal of Parasitic Diseases*, **23**: 125–128.
- Ranson, H., Abdallah, H., Badolo, A., Guelbeogo, W. M., Kera-Hinzoumbé, C., Yangalbé-Kalnoné, E., et al. 2009. Insecticide resistance in *Anopheles gambiae*: data from the first year of a multi-country study highlight the extent of the problem. *Malaria Journal*, **8**: 299, doi: 10.1186/1475-2875-8-299.
- Ranson, H., N'Guessan, R., Lines, J., Moiroux, N., Nkuni, Z., and Corbel, V. 2011. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends in Parasitology*, **27**: 91–98.
- Reyes-Villanueva, F., Juarez-Eguia, M., and Flores-Leal, A. 1990. Effects of sublethal dosages of Abate upon adult fecundity and longevity of *Aedes aegypti*. *Journal of the American Mosquito Control Association*, **6**: 739–741.
- Robert, L.L. and Olson, J.K. 1989. Effects of sublethal dosages of insecticides on *Culex quinquefasciatus*. *Journal of the American Mosquito Control Association*, **5**: 239–246.
- Rowland, M. and Hemingway, J. 1987. Changes in malathion resistance with age in *Anopheles stephensi* from Pakistan. *Pesticide Biochemistry and Physiology*, **28**: 239–247.
- Rust, M.K. 1995. Factors affecting control with residual insecticide deposits. In *Understanding and controlling the German cockroach*. Edited by M.K. Rust, J.M. Owens, and D.A. Reiersen. Oxford University Press, New York, New York, United States of America. Pp. 149–169.

- Sanil, D. and Shetty, N.J. 2012. The effect of sublethal exposure to temephos and propoxur on reproductive fitness and its influence on circadian rhythms of pupation and adult emergence in *Anopheles stephensi* Liston—a malaria vector. *Parasitology Research*, **111**: 423–432.
- Shetty, N.J. 1983. Chromosomal translocations and semisterility in the malaria vector *Anopheles fluviatilis* James. *Indian Journal of Malariology*, **20**: 45–48.
- Shetty, N.J. 1997. Genetic control of mosquito vectors of diseases. *Journal of Parasitic Diseases*, **21**: 113–121.
- Shetty, N.J. 2002. The genetic control of *Anopheles stephensi* - a malaria mosquito. In *Trends in malaria and vaccine research: the current Indian scenario*. Edited by D. Raghunath and R. Nayak. Tata McGraw-Hill, New Delhi, India. Pp. 44–79.
- Stark, J.D. and Banks, J.E. 2003. Population-level effects of pesticides and other toxicants on arthropods. *Annual Review of Entomology*, **48**: 505–519.
- Tan, Y., Biondi, A., Desneux, N., and Gao, X.W. 2012. Assessment of physiological sublethal effects of imidacloprid on the mirid bug *Apolygus lucorum* (Meyer-Dür). *Ecotoxicology*, **21**: 1989–1997.
- Vasuki, V. 1992. Adult longevity of certain mosquito species after larval and pupal exposure to sublethal concentration of an insect growth regulator, hexaflumuron. *Southeast Asian Journal of Tropical Medicine and Public Health*, **23**: 121–124.
- Windbichler, N., Papathanos, P.A., Catteruccia, F., Ranson, H., Burt, A., and Crisanti, A. 2007. Homing endonuclease mediated gene targeting in *Anopheles gambiae* cells and embryos. *Nucleic Acids Research*, **35**: 5922–5933.
- Windbichler, N., Papathanos, P.A., and Crisanti, A. 2008. Targeting the X chromosome during spermatogenesis induces Y chromosome transmission ratio distortion and early dominant embryo lethality in *Anopheles gambiae*. *Public Library of Science Genetics*, **4**: e1000291.
- World Health Organization. 2005. Guidelines for laboratory and field testing of mosquito larvicides. WHO/CDS/WHOPES/GCDPP/2005.13 Available from http://apps.who.int/iris/bitstream/10665/69101/1/WHO_CDS_WHOPES_GCDPP_2005_13.pdf [accessed 9 September 2016].
- World Health Organization. 2009. Recommended insecticides for indoor residual spraying against malaria vectors [online]. Available from www.who.int/whopes/Insecticides_IRS_Malaria_09.pdf [accessed 9 September 2016].
- World Health Organization. 2012. Factsheet on the world malaria report 2012 [online]. Available from http://www.who.int/malaria/media/world_malaria_report_2012_facts/en [accessed 21 October 2016].
- World Health Organization. 2013. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Available from http://apps.who.int/iris/bitstream/10665/80139/1/9789241505154_eng.pdf?ua=1 [accessed 21 October 2016].
- World Health Organization. 2015. Global Health Observatory (GHO) data. Available from http://www.who.int/gho/malaria/insecticide_resistance/en [accessed on 21 December 2015].
- Zaim, M. and Guillet, P. 2002. Alternative insecticides: an urgent need. *Trends in Parasitology*, **18**: 161–163.
- Zin, T. and Shetty, N.J. 2008. Sublethal effect of bifenthrin and neem on fecundity, hatchability and sex ratio of *Anopheles stephensi* Liston, a malaria mosquito. *Pestology*, **32**: 39–44.