International Journal of Mosquito Research

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2017; 4(2): 135-141

Received: 19-01-2017
Accepted: 20-02-2017

Vimla Thareja
Department of Zoology, Miranda House, University of Delhi New Delhi, Delhi, India

Anina James
Department of Zoology, Miranda House, University of Delhi New Delhi, Delhi, India

Rangoli Singh
Department of Zoology, Miranda House, University of Delhi New Delhi, Delhi, India

Anjana Singha Naorem
Department of Zoology, Miranda House, University of Delhi New Delhi, Delhi, India

Manisha Pandey
Department of Zoology, Miranda House, University of Delhi New Delhi, Delhi, India

Zeba Khan
Department of Zoology, Miranda House, University of Delhi New Delhi, Delhi, India

Souvik Sensharma
Reproductive biology, National Institute of Immunology New Delhi, Delhi, India

Stuti Mohapatra
Department of Zoology, Miranda House, University of Delhi New Delhi, Delhi, India

Vimla Thareja and Anina James are joint first authors.

Correspondence
Vimla Thareja
Department of Zoology, Miranda House, University of Delhi New Delhi, Delhi, India
E-mail: vt1218@gmail.com

Overwintering sites and strategies of *Culex quinquefasciatus* (Diptera: Culicidae) larvae and impact of neem seed kernel aqueous suspension (NSKAS) and other eco-friendly tools on them

Vimla Thareja, Anina James, Rangoli Singh, Anjana Singha Naorem, Manisha Pandey, Zeba Khan, Souvik Sensharma and Stuti Mohapatra

Abstract
Larval sites of *Culex quinquefasciatus* Say were discovered outdoor in urban environment, during winters (2006-2015). In these sites prolongation of the larval period to ~28 days was major overwintering strategy of larvae and 40% mortality was observed during larval period. Prolongation provided a long duration for elaborate study of their biological traits. Introduction of *Gambusia affinis* in one site showed its predatory role and induced deterrence to oviposition of *Cx.quinquefasciatus* during winter.
Effect of neem seed kernel aqueous suspension (NSKAS), concentrations 625-10,000 ppm was studied on overwintering fourth instar larvae under field conditions for two years. At low concentrations, effect at 24 h was sublethal; post 24 h manifestations exhibited as severe deformities and mortality. Treated larvae showed no antifeedancy, rectal prolapse, anal gills shrinkage and shedding, air bubbles around tracheae, convulsions, leading to mortality evidencing NSKAS as stomach, respiratory and nerve poison. Larval-pupal intermediates indicated moulting disruption. Use of NSKAS, *G. affinis* or source reduction of larvae are eco-friendly tools suggested for Integrated Vector Management (IVM) during winter, on priority basis.

Keywords: Deformities, NSKAS, overwintering, sublethal

1. Introduction
Overwintering of various stages of mosquito species in diverse habitats has been reported across the globe. For *Culex quinquefasciatus* Say, it has been documented for adults and larvae. The overwintering strategies of adults of this species has been reported by Reisen *et al.* [1], Ramaiah and Das [2], Almiron and Brewer [3], Nelms *et al.* [4], and Thareja *et al.* [5]; but less is known about overwintering strategies of their larvae. However, Hayes [6], in a study of isolated population of this species in Houston, observed that egg production was maintained by slow but constant emergence of adults despite snowfalls. He also reported highest mortality in the fourth instar larvae and prolonged life cycle in January. Almiron and Brewer [3] exposed lab reared females of same species to a range of 18.7 °C-8.92 °C in the fields in Argentina. The larvae that hatched from the eggs laid by these females, when exposed to a minimum temperature of 8.96 °C, showed high mortality and the period for immature stages was prolonged. Mogi [7] reported low survival rates of second instar larvae of same species and larval period prolongation when subjected to 10 °C and 10 h photoperiod. No comprehensive field study has been reported for overwintering strategies of larvae of this species. *Cx. quinquefasciatus* is a species of tropical and subtropical origin [8]. It is a major vector for lymphatic filariasis, a disease that has affected 119 million people living in 73 countries with India accounting for 40% of the global prevalence of infection [9]. This species is also known to spread viral diseases such as West Nile virus (WNV), St. Louis Encephalitis Virus (SLEV) and as a potential vector of Rift Valley Fever Virus (RVFV), Japanese Encephalitis Virus (JEV) and Zika virus. Therefore, need was felt to explore overwintering sites with a focus on overwintering strategies of the larvae of this medically significant species, knowledge of which may hold potential to curb their numbers during winter.

Our study included hunting larval sites to understand the habitat chosen by the females for oviposition in the urban environment during winter. It also includes removing larvae from the sites for source reduction and studying their behavioural, morphological, anatomical features and mortality. Impact of *Gambusia affinis* on this species of mosquito during winter was also explored.

Cx. quinquefasciatus breed most heavily in sewage water during optimal conditions [10], indicating their tolerance for toxicity of sewage water. Also, they are known to be resistant to a number of synthetic insecticides [11]. Such larvae are hardy and difficult to control. This highlights the need to control the larvae, preferably during winter when their numbers are low. Steps taken in this regard would prevent population explosion that occurs under optimum conditions. Hence, further study was conducted to understand the effect of biopesticide, Neem Seed Kernel Aqueous Suspension **(**NSKAS) on overwintering early fourth instar larvae collected from the sites and reared under outdoor conditions of winter. It included the impact of NSKAS on behavioural, morphological, anatomical features and survival of larvae. Neem is biodegradable and its seeds are most potent part of tree as a pesticide [12]. Its degradation is less at low temperature [13]; this provided an added advantage to the use of neem during winter.

2. Materials and Methods

2.1 Sites

They included urban natural larval sites which already existed and semi-natural sites created inside an educational institute in North Campus, University of Delhi, Delhi, India.

Existing natural sites - Surveillance was carried out weekly to locate larval sites in their natural habitat from November to March for 10 years (2006 to 2015) in and around the institute. All the existing sites discovered, were at ground level inside the institute and labelled as Site 1, Site 2 and Site 3. Site 1 was a pond made of concrete discovered in the year 2006, Site 2, a vase made of concrete discovered in 2007 and Site 3, a green plastic bucket discovered in 2010. Observations on the habitats of these sites were recorded. Site 1 was a major site in terms of its size and density of larvae and it existed since 1972. Incidentally, in this site high density of larvae was observed during monsoon (August), 2006. Water from this site was flushed out to remove the larvae and replenished with fresh water. The site was screened for larvae during winter (2006-07). In autumn 2007, five *G. affinis* were introduced into this site. It was again checked for egg rafts and larvae during winter, the following year (2007-08). In 2008, the institute authority decided to break this site and construct a new pond approximately 50 m away from it. Contents of this pond along with *G. affinis* (number increased from 5 to ~100) were transferred to the newly constructed pond. Presence of egg rafts and larvae was checked in the new pond during winter (2008 to 2015). The new pond exists till date.

Asynchronous culture - The larvae collected from Site 1, Site 2 and Site 3 were segregated as per instars and maintained separately for these sites under outdoor conditions. They were fed with powdered dog biscuit and yeast in ratio 3:1. Food was changed every alternate day till they transformed into pupae. Observations relating to overwintering were recorded in these larvae.

Created semi-natural sites: They included Site 4 and Site 5 created under outdoor conditions. Site 4 was represented in the form of four replicas; they were white enamel bowls containing tap water placed at safe and approachable corners both at ground and first floor every week during winter (2008 to 2015) within the institute. The total number of replicas was ~290. Site 5 was a mesh cloth cage, created according to the availability of blood fed females from the adult site (Thareja *et al.* [5]). This site could be created six times per year during winter (2011-12 and 2012-13). Females collected for Site 5 were provided with 5% sucrose as food and bowl of water for oviposition. Egg rafts laid in the range of 10 h in Site 4 and Site 5 were kept in outdoor conditions for hatching.

Synchronous culture: These larvae showed synchrony and were reared under outdoor conditions, in the same way as asynchronous culture till pupation.

Total number of existing and created sites was ~300.

Natural population of larvae from the existing natural sites (Site 1, 2 and 3) and field population from the created semi-natural sites were used for the study of overwintering strategies as one part of study.

Lack of availability of overwintering larvae in some of the years and their insufficient numbers in other years limited the study of effect of NSKAS on the larvae to winters of 2010-11 and 2013-14. In these two years only Site 4 gave us the number just sufficient to carry out this study.

2.2 Preparation of NSKAS and larval treatment

Neem seed kernels were washed gently with tap water, dried in shade at room temperature and powdered in electric blender. NSKAS stock solution of 10,000 ppm was prepared by dissolving powdered kernels in distilled water. From stock solution, concentrations 625, 1250, 2500, 3750, 5000, 7500 and 10,000 ppm were prepared and used for treatment. Overwintering late third/early fourth instar larvae from synchronous culture of Site 4 were used to understand the effect of NSKAS. WHO [14] protocol was followed for the treatment. 10 larvae were transferred into a petridish containing 20 ml of desired concentration and kept for 24 h. Control was prepared using 20 ml distilled water with same number of larvae as experimental. Overwintering larvae were considered as control. After 24 h treatment, the medium was replaced with water. There were 3 replicates each for experimental and control set ups and number of larvae obtained were just sufficient to run the experiment.

2.3 Study of characteristics of Control and NSKAS treated larvae

After treating the larvae with NSKAS for 24hs, observations of behavioural, morphological and anatomical characteristics were recorded, at 24 h and post 24 h (without NSKAS) using light microscopy, in 2010-11 and 2013-14. For control, similar observations were recorded without using NSKAS. Also, mortality of larvae for each concentration was recorded at 24 h and post 24 h till they became pupae. Total larval mortality was calculated by adding mortality at 24 h and mortality post 24 h. Thorax of test larvae which did not show movement were touched gently with a needle. Those that showed no signs of movement even after prodding were considered dead while those that showed very slow movement, which did not persist were considered moribund. While calculating mortality, moribunds were counted as dead.

2.4 Statistical Analysis
The data was analyzed using Graphpad Prism 5. Larval mortality at different concentration of NSKAS was analyzed using two-way ANOVA with two factors, mortality at 24 h and total mortality. Comparison of means was done by Bonferroni's Multicomponent Analysis. Probit analysis was done for calculating LC_{50} [15]. Probits were subjected to linear regression analysis to obtain correlation coefficient. All tests were done at $P<0.05$.

3. Results
Winter (December-March) temperature in Delhi usually ranges from 22 °C to 5 °C. During our 10 years study period (2006-2015) lowest temperature recorded was 1.9 °C, in the year 2013. Generally, winter here is drier as compared to rest of the seasons and has a photoperiod of 10:14 (L: D).

3.1 Sites
Existing natural sites - Site1, Site 2 and Site 3 contained stagnant water. These sites were open and sun-exposed with no overhung vegetation and located at ground level. Ground on which they were located was covered with green and dried patches of short grass. There was rich vegetation in and around the institute. The area where the sites were present was enclosed within two to three walls of 3 meters height. Egg rafts and larvae were observed in these sites. None of the sites had intra- and inter-specific competitors or predators during winter but in Site 1 *G. affinis* was introduced later. The detailed description of the sites follows.
Site 1: It was a pond (l-3 x b-1.8 x h-1m) located in Botany garden of the institute. It was filled with groundwater and contained *Hydrilla* introduced for maintaining the site. Plant litter got collected on the surface and within the site from time to time. As mentioned in materials and methods, flushing of water from this site during monsoon reduced the number of larvae in it. Distribution got further sparse during winter (2006-07). After introducing *G. affinis* to this site no larvae were observed. Thereafter no egg rafts or larvae were observed during winter (2007-08). Similar results were observed in new pond (also labelled as Site 1; dimensions same as old pond) during winter (2008 to 2015). The study could not be carried out in this site from 2007 to 2015 due to the absence of egg rafts and larvae during winter.
Site 2: It was a vase (l-0.6 x b-0.5 x h-0.38 m), filled with ground water. Its surface was covered with plant litter and contained approximately 500 larvae consisting of second, third and early fourth instar. It was observed in the first week of December 2007, at a distance of 7 meters from Site 1in the same garden.
Site 3: It was a bucket (r-20cm, h-45cm). It contained water that dripped out of the air conditioner (AC) of the library building located in an isolated corner within the institute. It contained plant litter on the surface and approximately 600 larvae consisting of second, third and early fourth instar. This site was observed in last week of November, 2010, at a distance of ~ 100 meters from Site 1.
Created semi-natural sites
Site 4: Its replicas were bowls (r-12 cm, h-15 cm). Although artificial, it acted as natural site for females to oviposit. Egg rafts were laid in the bowls kept on both floors. The total number of eggs in an egg raft ranged from 35 to 150. As mentioned in materials and methods, sufficient number of eggs leading to synchronous culture was available only for two years (2010-11 and 2013-14) from this site. Larvae from this culture were used for studying overwintering and effect of NSKAS treatment.
Site 5: The site comprised a cage (l-0.3 x b-0.3 x h-0.3m). Although artificial it acted as semi-natural site for blood fed females to oviposit. Number of eggs per egg raft laid in this site ranged from 65 to 150. This site did not give sufficient number of eggs at a time; therefore, larvae from this site were used only for studying overwintering.

3.2 Characteristics of control larvae
The larvae were large sized and less tanned. They were frequently seen moving to lower layers of water. They were sluggish in movement even as their feeding brushes moved actively. In some larvae, darkening of tracheal tubes was observed. Larval period in fourth instars was prolonged to maximum of 10 days and the total larval period, to a maximum of 28 days. Larvae showed maximum of 40% mortality during their total larval period.

3.3 Characteristics of NSKAS treated larvae
Larvae showed restlessness as an immediate response to NSKAS treatment. Their mouth brushes showed active movement. Some larvae were observed frequenting upper layers of the medium with siphon pointing up. Coiling of larvae and auto-grooming of spiracle and surface of siphon by aggressive biting was observed. Some showed convulsions and others, a gradual slowing down of movements. In both cases the larvae became either moribund or dead and the number of such larvae increased with increase in the concentration of NSKAS. Some larvae were observed in unnatural reverse posture with ventral side up, these larvae later died. The treated fourth instar larvae showed extension of the already prolonged larval period by maximum of 4 days at low concentrations of NSKAS (625 ppm, 1250 ppm). At high concentration, larvae died before 10 days.
Treated larvae showed several morphological deformities on head, thorax and abdomen. They showed irregular pigmentation throughout the body, particularly on head. In some larvae, ocelli were distorted. Loss of tissue was observed in and around the ocelli and compound eyes. Some larvae showed stretched arthrodial membrane, most prominent between head and thorax; muscles in this area also showed stretching. Thorax of most larvae had bulbous appearance. Tracheal tubes showed darkening and constrictions in the head, thorax and abdomen. In some larvae there was accumulation of air bubbles around these constrictions; in others, tracheae ruptured and in still others they were shredded, particularly in thoracic region. In some cases, rectal prolapse was observed between anal gills (Fig. 1). Shrinkage and irregular pigmentation in the anal gills was observed (Fig. 1), followed by dense pigmentation in them. In rare cases shedding of the gills was observed closer to death of larvae. Larval-pupal intermediates (Fig. 2), observed at concentrations ≥2500, died as soon as they were formed.
In 2010-11, larval mortality at 24 h was equal or higher than that observed post 24 h except at the lowest concentration, 625 ppm, for which mortality was observed only post 24 h (Fig. 3). The dose dependent increase in mortality was observed at 24 h and for total fourth instar larval period ($P<0.05$). Mortality at 24 h ranged from 40% to 68% for

concentrations ≥ 1250ppm and total larval mortality ranged from 30% to 95% for concentrations ≥ 625ppm. LC_{50} for total larval mortality was 657 ppm (10 ppm to 1462 ppm; R^2=0.85; P=0.02; Slope= 1.6±0.3; χ^2= 0.0004; F = 1.87; DFn =5; DFd = 14).

In 2013-14, larval mortality was observed at both 24 h and post 24 h in all the concentrations of NSKAS except at lower concentrations, 625 ppm and 1250 ppm (Fig. 4). Mortality at 24 h for all concentrations was equal or higher than observed post 24 h, except at 3750ppm, at which mortality was observed only post 24 h. The dose dependent increase in mortality at 24 h was observed for concentration 5000 ppm and onwards and for total fourth instar larval period, it was 3750 ppm and onwards (P<0.05). Mortality at 24 h for concentrations ≥5000 ppm ranged from 27% to 53%, and for concentrations ≥ 3750ppm total mortality ranged from 23% to 77%. LC_{50} for total larval mortality was 4753 ppm (4092 ppm to 5407 ppm; R^2=0.99; P=0.003; Slope= 2.4 ± 0.1; χ^2= 0.000028, F= 4.85; DFn = 6; DFd=14).

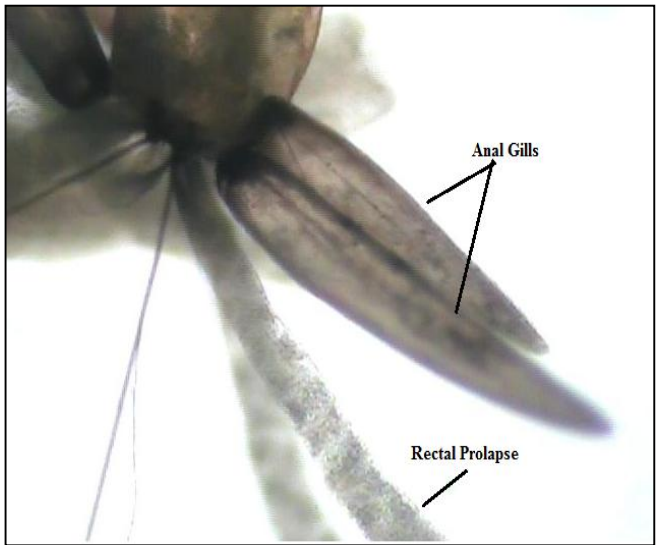

Fig 1: Rectal prolapse and pigmented anal gills in treated larvae

Fig 2: Larval-pupal intermediate

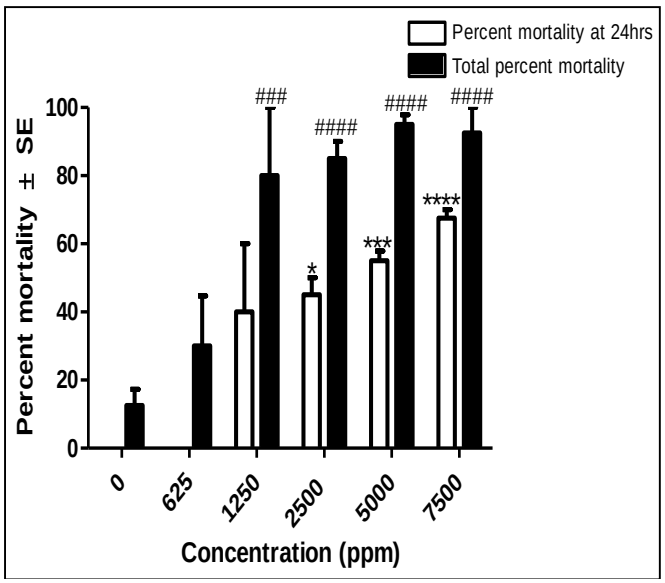

Fig 3: Larval mortality at 24 h and total larval mortality after treatment with NSKAS in 2010-2011. Significant differences (P<0.05) between means when compared with control are denoted using * for mortality at 24 h and # for total mortality.

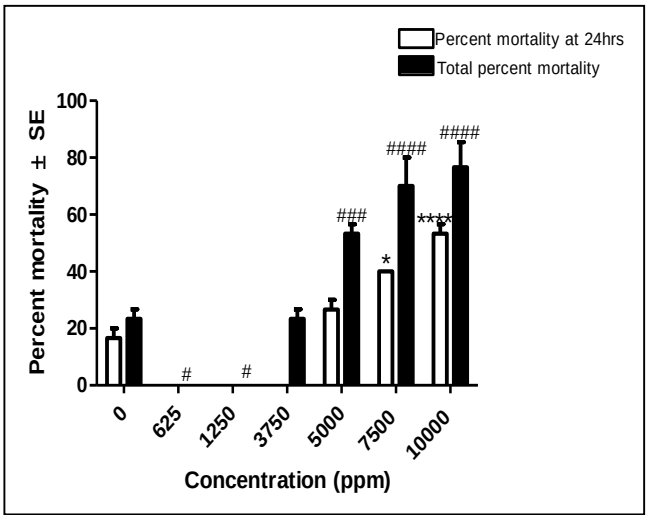

Fig 4: Larval mortality at 24 h and total larval mortality after treatment with NSKAS in 2013-2014. Significant differences (P<0.05) between means when compared with control are denoted using * for mortality at 24 h and # for total mortality.

4. Discussion

To the best of our knowledge, present research is the first field study on overwintering sites and strategies adopted by *Cx. quinquefasciatus* larvae in an urban environment in Indian perspective. Larval breeding sites are very rare in winter particularly in the urban scenario; nevertheless, after extensive survey we discovered some sites that were man-made habitat and man-made container habitats [16]. The sites which we created were man-made container habitats. The sites were chosen by the ovipositing females possibly because of absence of predators and competitors and presence of other favorable conditions. As a part of our study, removing the sites or larvae from the sites meant source reduction. Almiron and Brewer [17] reported the frequent presence of *Cx. quinquefasciatus* larvae in container habitats during optimum conditions. In our study, clear and stagnant water in the sites

provided stable and healthy environment to immatures and ovipositing females, which are metabolically low during winter. However, larvae could survive in Site 3 which contained AC water. Sussman and Portnoy [18] have reported the presence of water soluble impurities in AC water. Therefore, our study reveals the ecological plasticity of larvae for clear and polluted water during winter. Forattini [19] reported ecological plasticity this species under optimum conditions. Hence this plasticity is relevant for both optimum and winter conditions for larvae of this species. Our study also reveals that females chose to oviposit not only at ground level but also at a height of 4 meters even when they were in a low metabolic state. While these females were active enough to oviposit, Thareja *et al.* [5] reported of other females that were passive and opted to overwinter in adult sites and did not quit those sites for oviposition for a maximum period of 3 months despite being gravid.

There was rich vegetation within and outside the institute and around the sites which provided natural habitat for the females. Absence of overhung plants on the larval sites ensured sun exposure and walls enclosing the sites reduced the effect of cold wind. These factors perhaps prevented extreme drop in temperature providing suitable environment for oviposition. Hayes [6] reported that water temperature was one of the foremost factors deciding larval survival and development. According to Grech *et al.* [20] fallen plant leaves provided nourishment and protection to the developing larvae against outside predators and physical disturbances such as wind. In our study, plant litter in the sites must have served the same purpose.

Winter conditions impacted the larvae, in which some overwintered and others died. During the larval period, ~ 40% mortality in each site was observed. Absence of larvae in Site 1 after introducing *G. affinis* in it possibly indicated the predatory role of *G. affinis*. Thereafter absence of egg rafts and larvae in this site in following winters (2007 to 2015) suggested deterrence to oviposition. Angelon and Petranka [21] reported similar observations on *Culex* sps. and demonstrated that ovipositing females can use chemosensory mechanisms to assess predator risk to offsprings in the outdoor conditions of July in Mecklenburg County, North Carolina. In our case similar kind of mechanisms must have operated in ovipositing females during winter.

Decision of choosing sites for oviposition lie with the blood-fed females. These are the sites which are later established as larval sites. In our study, females had limited choice of natural sites for oviposition because of limited rains during winter and constraints of the urban environment. Sites with stagnant water confined the larvae, preventing their migration and compelling them to acquire adaptations during winter. One such adaptation was exhibited in the form of frequenting of larvae to lower layers of water. Such a behavioral adaptation acquired must have been for attaining warmth. Prolongation of the larval period to a maximum of 28 days was another adaptation a major overwintering strategy and was of significantly long duration, when compared with the 8 days life cycle reported for optimum conditions [22]. Prolongation was also reported by Hayes [6] and Almiron and Brewer [3] but for immature stages of *Cx. quinquefasciatus*. Hayes [6] reported this prolongation in isolated population, during snowfall. On the other hand, Almiron and Brewer [3] observed prolongation, when lab reared females were exposed to a temperature range of 18.7 °C-8.92 °C and the larvae emerged were exposed to 8.96 °C in the field. Adoption of prolongation of larval period, perhaps allowed the larvae to acquire enough energy to beat temperature as a stressor and to meet the requirement for molting. Prolongation must have led to their large size and sluggishness and delay in the molting. These overwintering larvae, though sluggish yet were active when compared with the immobile overwintering adults reported by Thareja *et al.* [5]. During larval development, temperature and photoperiod are the two important variables of weather known to impact growth, development rate and survival. Mogi [7] showed that providing 10 °C and 10 h photoperiod approximated the threshold of larval development. In our study photoperiod was closer to 10h and its variations were minor whereas, temperature variations from 22 °C to 5 °C during this period were major; hence we presume that most of the changes observed in the larvae were more as repercussions of temperature than photoperiod. Prolongation of larval period and high mortality of the larvae during winter imply low number of adults and thus less number of life cycles, resulting in natural regulation of their population during winter. Winter is thus the appropriate period for intervention by eco-friendly tools to achieve effective larval control. On the other hand, prolongation of larval period would benefit survival of the viruses, which if present, can be vertically transmitted. Goddard *et al.* [23] reported the vertical transmission of West Nile Virus in *Cx. quinquefasciatus*. Curbing the larvae population during winter would prevent the viral transmission.

So far there have been no reports on the effect of pesticides on overwintering larvae of *Cx. quinquefasciatus* during winter. Our study is the first report on NSKAS treatment on overwintering larvae of this species. NSKAS impacted their biological traits such as behavior, morphology and anatomy and led to sublethal and lethal effects in them. At low concentrations of NSKAS, 625 ppm for 2010 and 625 ppm, 1250 ppm for 2013, extension of the already prolonged larval period indicated further delay in moulting. This period provided a long duration for extensive study of behavioral, morphological and anatomical features. The behavioral changes in treated larvae involving their frequenting upper layers of water and aligning their body such that spiracles located on the siphon came in contact with outside air possibly exhibited avoidance of the pesticide in the medium. During this process the larvae got exposed to low temperature of upper layers which must have affected their survival. In others, behavioral changes exhibited in the form of aggressive self-biting and auto-grooming of the respiratory siphon might have been for clearing the clogged spiracles and siphon surface so as to maintain its stability and steady flow of air. Larvae post treatment seemed more sluggish than untreated larvae, making them more vulnerable to the harsh environment. The deformities such as irregular pigmentation on head and, stretched neck muscles and arthrodial membrane implied improper growth, thereby further exposing them to adverse environmental conditions. Degeneration of tissue in and around the ocelli and eyes is suggestive of the effect of NSKAS on photosensitivity of ocelli and vision of eyes and its role as nerve poison. Reverse posture observed in some larvae could have been the impact of impaired neuromuscular coordination that gradually led to their death; this further evidenced NSKAS as nerve poison. Another morphological

deformity observed in some larvae was bulbous thorax; this must have been due to fluid retention. Such larvae could not survive. Further observations revealed that NSKAS also affected anatomy of tracheal system. Darkening of tracheal tubes could have been due to hypoxia. Deep constrictions that appeared in the walls of tracheae in head, thorax and abdomen of treated larvae probably resulted in weakening of the walls, leading to leakage of air and formation of air bubbles around the constrictions. This weakening might have led to rupturing and shredding of the tracheal tubes. Such impairment would surely interfere with the physiological functioning of the respiratory system leading to mortality of larvae, indicative of NSKAS as a respiratory poison. Shrinkage and pigmentation of anal gills were probably due to disruption of osmotic and ionic balance. Such deformities of the gills seem to have led to their fragility and shedding when close to death. NSKAS did not act as an antifeedant even at the highest concentration; this was evidenced by the active movement of feeding brushes. Rectal prolapse in the larvae is suggestive of the effect of NSKAS on posterior part of alimentary canal, further confirming that it was not an antifeedant but a stomach poison. Above mentioned deformities must have hampered the physiological functions and their severity ultimately led to mortality. The formation of larval-pupal intermediates indicated the inhibition of pupation possibly due to molting disruption which led to mortality. Our observations suggest that NSKAS is efficacious at low temperature.

The water soluble, active ingredients of neem seeds, Azadirachtin, the most active of all [24], induced larval mortality not only during 24 h but its effect manifested post 24 h, even at low concentrations thereby exhibiting its long term effect. The mortality till 24 h must have been due to the cumulative effect of the two stressors, winter conditions and NSKAS. But, post 24h was possibly due to winter conditions and manifestations of NSKAS when NSKAS was not in the medium. Dose dependent increase in mortality at 24 h and mortality for total fourth instar larval period was observed. Total larval mortality close to 100% was achieved at 5000 ppm (2010-11) and 10,000 ppm (2013-14). Martínez-Tomas *et al.* [25] reported 100% mortality at 1000 ppm of aqueous neem seed extract, in *Cx. quinquefasciatus* larvae reared in the insectary conditions at 27 °C in Mexico. We studied the effect of NSKAS, on larvae by rearing them under winter conditions when temperature was in the range of 4 °C to 10 °C under outdoor conditions, which is 23 °C to 17 °C lower than the temperature (27 °C) at which larvae were treated in the study conducted by Martínez-Tomas *et al.* [25]. In our study, low temperature during winter could be the possible reason for the requirement of higher concentration of NSKAS to achieve the mortality close to 100%. Similar opinion has been expressed by Muturi *et al.* [26] for malathion treatment of *Cx. restuans* larvae at 20 °C where low temperature buffered against some of the negative effects of insecticide. Besides low temperature, wild population could be the other cause for requirement of high concentration of the pesticide to achieve the mortality close to 100%. The above said two factors, winter conditions and wild population caused variation in LC_{50} value for 2010-11 when compared with LC50 value for 2013-14.

Neem technology has been prevalent in Asia, Africa, central and south America where neem trees are native and also in parts of United States, Canada, Europe and Australia where neem tree farming is established. In view of global availability of neem, NSKAS based application proposed in the present study will have no constraint in its large scale implementation world over. This basic technology would provide sustainable vector control programme, which is in line with the requirements WHO has listed for Integrated Vector Control programme.

5. Conclusion

We discovered overwintering larval sites of *Cx. quinquefasciatus* in an urban scenario. In these sites, prolongation of larval period to ~28 days was a major overwintering strategy. This strategy might have enabled this species to expand its geographical niche and develop endemicity, should the climate change occur. The prolongation provided a long window for elaborate study of its biological traits. Exposure of fourth instar overwintering larvae to NSKAS extended the already prolonged larval period, thereby providing a longer window for study of morphological and anatomical deformities in a sequential manner without missing details. The deformities and mortality were on account of multiple modes of action such as stomach, respiratory and nerve poison. Even at low concentration of NSKAS, targeted high mortality could be attained, though the effect during 24h was sub-lethal due to low concentration of NSKAS in the medium and post 24h, only due its manifestation leading to severe deformities. Neem acted as a potent bio-pesticide even during winter. In view of global availability of neem, application of this basic NSKAS based technology will have no constraint in its large scale implementation world over. *G. affinis* served as another eco-friendly tool for larval control, because its introduction in one site showed its predatory role and induced deterrence to oviposition of *Cx.quinquefasciatus* during winter. Thus source reduction by removing the larval sites, using NSKAS or using *G. affinis* when the larvae are in low metabolic state and low in number, would restrict the pesticide resistant winter hardies much before its population explosion post winter, and curb the spread of viruses and filariasis. These affordable eco-friendly tools are suggested to be made a part of Integrated Vector Management (IVM) during winter on priority basis. Special attention should be focused on its control strategies, in case this potent vector of RVFV, JEV and Zika virus becomes a functional vector.

6. Acknowledgements

We highly acknowledge Prof. M.K.K. Pillai, ex-consultant of UNEP, for discussions relating to the subject of the paper; Prof. Nadeem Ahmad (Jamia Milia Islamia University, Delhi) for discussions relating to statistical analysis; anonymous reviewers, whose suggestions helped us in improving quality of our manuscript; Sarika, Swati Bansal, Swati Malik, Reenu (BSc III year, Zoo. major) Garvita, Kanika, Shruti, Radhika (BSc. I year, Zoo. Major and I L.S.) for assisting the corresponding author with some parts of the investigations; Principal, Miranda House, for providing the infrastructure and the Lab staff of Zoology department, Miranda House, University of Delhi for their lab assistance.

7. References

1. Reisen WK, Meyer RP, Milby MM. Overwintering studies on *Culex tarsalis* (Diptera: Culicidae) in Kern

County, California: temporal changes in abundance and reproductive status with comparative observations on *C. quinquefasciatus* (Diptera: Culicidae). Annals of the Entomological Society of America, 1986; 79(4):677-685.
2. Ramaiah KD, Das PK. Seasonality of adult *Culex quinquefasciatus* and transmission of bancroftian filariasis in Pondicherry, South India. Acta Tropica. 1992; 50(4):275-283.
3. Almirón WR, Brewer ME. Winter biology of *Culex pipiens quinquefasciatus* say,(Diptera: Culicidae) from Córdoba, Argentina. Memórias do Instituto Oswaldo Cruz. 1996b; 91(5):649-654.
4. Nelms BM, Macedo PA, Kothera L, Savage HM, Reisen WK. Overwintering biology of *Culex* (Diptera: Culicidae) mosquitoes in the Sacramento Valley of California. Journal of Medical Entomology. 2013; 50(4):773-790.
5. Thareja V, Singh R, Naorem AS. Some observations on overwintering sites of adult *Culex quinquefasciatus* (Diptera: Culicidae) and strategies followed under natural and seminatural conditions. Parasitology Research. 2016; 115(1):195-203.
6. Hayes J. Seasonal Changes in Population Structure of *Culex pipiens quinquefasciatus* Say (Diptera: Gulicidae): Study of an isolated population. Journal of Medical Entomology. 1975; 12(2):167-178.
7. Mogi M. Temperature and photoperiod effects on larval and ovarian development of New Zealand strains of *Culex quinquefasciatus* (Diptera: Culicidae). Annals of the Entomological Society of America. 1992; 85(1):58-66.
8. Vinogradova EB. *Culex pipiens pipiens* mosquitoes: taxonomy, distribution, ecology, physiology, genetics, applied importance and control. Pensoft Publishers, Sofia, Bulgaria. 2000.
9. Ramaiah KD, Das PK, Michael E, Guyatt HL. The economic burden of lymphatic filariasis in India. Parasitology Today. 2000; 16(6):251-253.
10. US. Department of Health and Human Services. Mosquitoes of public health importance and their control. CDC Manual 6, Communicable Disease Centre, Atlanta. 1987.
11. Kumar K, Sharma AK, Kumar S, Patel S, Sarkar M, Chauhan L. Multiple insecticide resistance/susceptibility status of *Culex quinquefasciatus*, principal vector of bancroftian filariasis from filaria endemic areas of northern India. Asian Pacific Journal of Tropical Medicine. 2011; 4(6):426-429.
12. Schmutterer H. The neem tree, *Azadirachta indica* A. Juss. and other meliaceous plants: source of unique natural products for integrated pest management, medicine, industry and other purposes. VCH, Weinheim, Germany. 1995.
13. Schmutterer H, Ascher KRS. Natural pesticides from the neem tree (*Azadirachta indica* A. Juss) and other tropical plants. Proceeding of the Third International Neem Conference, Nairobi, Kenya. Eschborn, Germany. 1987.
14. World Health Organization. Guidelines for laboratory and field testing of mosquito larvicides. Communicable disease control, prevention and eradication. WHO pesticide evaluation scheme. WHO, Geneva. 2005.
15. Finney DJ. Probit analysis. Cambridge University Press, Cambridge. 1971.
16. Service MW. Medical Entomology for Students. Vol.3. Cambridge University Press, Cambridge, United Kingdom. 2003.
17. Almirón WR, Brewer ME. Classification of immature stage habitats of Culicidae (Diptera) collected in Córdoba, Argentina. Memórias do Instituto Oswaldo Cruz. 1996a; 91(1):1-9.
18. Sussman S, Portnoy IL. Water Composition Changes in Air-Conditioning Equipment. Journal of American Water Works Association. 1959; 51(8):953-963.
19. Forattini O. Culicidologia médica: identificação, biologia e epidemiologia, EDUSP, São Paulo, Brazil. 2002, 2.
20. Grech M, Sartor P, Estallo E, Ludueña-Almeida F, Almirón W. Characterisation of *Culex quinquefasciatus* (Diptera: Culicidae) larval habitats at ground level and temporal fluctuations of larval abundance in Córdoba, Argentina. Memórias do Instituto Oswaldo Cruz. 2013; 108(6):772-777.
21. Angelon KA, Petranka JW. Chemicals of predatory mosquito fish (*Gambusia affinis*) influence selection of oviposition site by *Culex* mosquitoes. Journal of chemical ecology. 2002; 28(4):797-806.
22. Shelton RM. The effect of temperatures on development of eight mosquito species. Mosq. News. 1973; 33(1):1-12.
23. Goddard LB, Roth AE, Reisen WK, Scott TW. Vertical transmission of west nile virus by three california *Culex* (Diptera: Culicidae) species. Journal of Medical Entomology. 2003; 40(6):743-746.
24. Mordue (Luntz) J, Nisbet AJ. Azadirachtin from the neem tree *Azadirachta indica*: its action against insects. Anais da Sociedade Entomológica do Brasil. 2000; 29(4):615-632.
25. Martinez-Tomas SH, Perez-Pacheco R, Rodríguez-Hernández C, Ramírez-Valverde G, Ruiz-Vega J. Effects of an aqueous extract of Azadirachta indica on the growth of larvae and development of pupae of *Culex quinquefasciatus.* African Journal of Biotechnology. 2009; 8(17):4245-4250.
26. Muturi EJ, Lampman R, Costanzo K, Alto BW. Effect of temperature and insecticide stress on life-history traits of *Culex restuans* and *Aedes albopictus* (Diptera: Culicidae). Journal of Medical Entomology. 2011; 48(2):243-250.