J Vector Borne Dis 44, December 2007, pp. 245–249

Morphological method for sexing anopheline larvae

S.N. Emami[a], H. Vatandoost[a], M.A. Oshaghi[a] , F. Mohtarami[a], E. Javadian[a] & A. Raeisi[b]

[a]*Department of Medical Entomology & Vector Control, School of Public Health & Institute of Health Research, Medical Sciences/Tehran University, Tehran;* [b]*Malaria Department, Ministry of Health & Medical Education, Tehran, Iran*

Abstract

Background & objectives: Most of autocidal control of malaria vectors relies on the rearing and release of large numbers of sterile male into a wild population and it would be crucial to separate the males from females before release. This could result in enormous economic benefits in the mass rearing and raise the efficiency of the field operations. The development of genetic sexing of mosquitoes, enabling the release of males only, but impairing the overall fitness of the released insect has been considered greatly. Here we report on a morphological sexing method for the preferential diagnosis and separation of males in late III and IV instar larvae for the mosquitoes *Anopheles stephensi* Liston and *An. culicifacies s.l.* (Diptera: Culicidae), the principal vectors of human malaria in Asia and Indian subcontinent.

Methods: Male mosquitoes are identified by their tube like organ at the 9th abdomen segment which originates from segment parallel to the spiracles. Length and width of this organ is measured as 66.66 ± 9.5 and 14.3 ± 1.5 µm respectively. The whole length of the organ is 201.63 ± 23.4 µm. Two fried eggs in the anterior portion of the segment are apparent in males. The length of tube in female is shorter than the male (almost half of the length–37.95 ± 4.0 µm), its width is slightly stout and wider than the male (16.72 ± 1.4 µm). Two fried eggs in the anterior portion of the segment are absent. After separation of live male larvae by those characteristics, they were transferred into the trays and emerged adults were identified to ascertain correct identification of sex.

Results: All the larvae with male organs developed into male adults with hairy antennae and club shaped palpi, whereas all the female larvae developed into adult females.

Interpretation & conclusion: The sex separation at the larval stage will provide a clue for embryonic origin of sex organs, insecticide selection at the larval stage, sex related genes, male sterility and other measures.

Key words *Anopheles* larvae – malaria – sex determination

Introduction

The sterile insect technique (SIT) is currently being used for the control of some medical and agricultural pests. The SIT relies on the rearing and release of large numbers of genetically sterile insects into a wild population. In many applications of autocidal control, it would be efficient to separate the males and females before release[1]. Avoiding assortative mating and any increase in the size of the natural population, and elimination of females which may be disease vectors or which cause damage to produce or livestock are

some possible uses of sexing for genetic control operations[2]. If one sex could be eliminated in the egg stage (pre-zygotic sexing) or larval-pupal stage (post-zygotic sexing) it could result in enormous economic benefits in the mass rearing and increase the efficiency of the field operations several fold[1,3]. In this case, twice as many insects (of one sex) could be produced with a given expenditure for diet and labour.

A number of schemes have been proposed to accomplish removal of females at the egg or early larval stage. In some species, alleles resistant to specific toxic chemicals, such as ethyl alcohol, endrin, purine, potassium sorbate, dieldrin, cyromazine, and propoxur have been selected. When the toxic substance is introduced into the colony, only the sex carrying the resistant allele survives. The most successful efforts for removal of females during the rearing process have been made with the mosquito, *Anopheles albimanus* Wiedemann (Diptera: Culicidae)[4,5] , the medfly, *Ceratitis capitata* Wiedemann[1,6], and the stable fly, *Stomoxys calcitrans* (L.)[7]. In addition, the development of transgenic sexing lines have been reported for the principal vectors of human malaria such as *An. stephensi* Liston[3,8], *An. culicifacies s.l.*[2], and *An. quadriannulatus s.s.*[9]. However, genetic changes could lead to decreased fitness in the manipulated population and reduce the efficiency of the SIT programme.

There are several characteristics for species identification of anopheline mosquito larvae, for instance tergal plates, palmate hairs and mesothoracic pleural hair. However, there is no published document on sex differentiation of anopheline larvae. Here we describe the special internal characteristics which can easily differentiate the male and female larvae of *Anopheles*. *An. stephensi* Liston and *An. culicifacies s.l.* have been the main malaria vectors in Iran[10]. The sexing normal/wild mosquito lines described here combine most of the features desired and required for a safe separation of males from females for application of sterility methodologies to malaria control programmes.

Material & Methods

Larval sample collections were carried out in two phases during April–June and September–November 2005 monthly. Larvae were sampled using standard dippers (500 ml) from breeding places close to adult collection sites. Larvae were carefully transported alive to the insectary and were reared to get adults. Adult collections were made from various habitats like pit shelters, domestic places, indoors of human and animal shelters, etc. by hand catch method using aspirator and torch light. All the specimens including blood fed or unfed mosquitoes caught from field were transported alive to the insectary. All the unfed females were kept in cages and fed on Guinea pig. Blood fed female mosquitoes were transferred individually into separate glass tubes (15 cm length and 2 cm diam) lined with wet filter paper for egg laying. Morphological characteristics of 20 larvae at late III or early IV instars from each single female cultures were analysed under compound microscope. Measurements were carried out using an ocular micrometer in a stereoscopic microscope at a magnification of 15X. The ocular micrometer was calibrated at regular intervals. The samples were identified morphologically using the pictorial key to Iranian anophelines to distinguish the *An. stephensi* Liston and *An. culicifacies s.l.* adults[10].

Determination of the sex of individual larva was based on identification of developing male and female secondary reproductive structures within the anal (9th) segment (Figures of male secondary reproductive structures in Chambers[11]). Females could be easily distinguished by observing the female genitalia and the absence of male internal genital parts. For female the length of tube was also measured to differentiate it from male apparently. A pipette was used to place the larvae on a Neubauer slide. This help in chilling and mounting the alive larvae on to slide for appropriate transparency and observation. This type of slide is useful to reflex the light for observing clearly the internal genital organs of

anopheline larvae. It is important to mount larva without injury, because the internal structures cannot be seen if turgor is not maintained. After most of the water was blotted away, a fine sable water colour brush (size 0.0) was used to gently position the larva ventral side up with the anal segment extended over the respiratory siphon. The anal segment was then examined at 40X under a compound microscope. Measurements were done using an ocular micrometer in a stereoscopic microscope at a magnification of 15X. After separation of alive male and female larvae by those characteristics, they were transferred into the trays and emerged adults were identified to ascertain the correct sex. Male adults with hairy antennae and club shaped palpi were separated from females.

Results & Discussion

According to Chambers[11] and based on our findings the male and female genital structures can be identified by focusing down through the ventral cuticle and the sclerotised saddle that wraps around all but mid-ventral portion of the segment. The rudiments of the gonocoxite, ejaculatory duct, and vas deferens are discernable under the cuticle. In the late III and early IV instars they have the appearance of two fried eggs in the anterior portion of the segment. They may be observed in the middle (Figs. 1 & 2) or may be

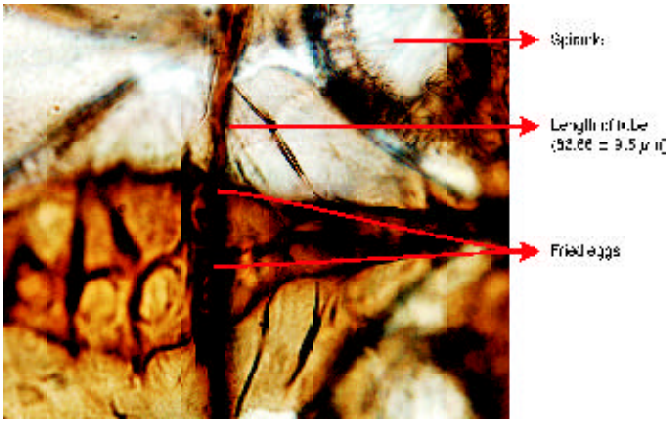

Fig. 1: Internal organ of male genitalia located in the 9th segment of abdomen which is parallel to spiracle of anopheline larvae

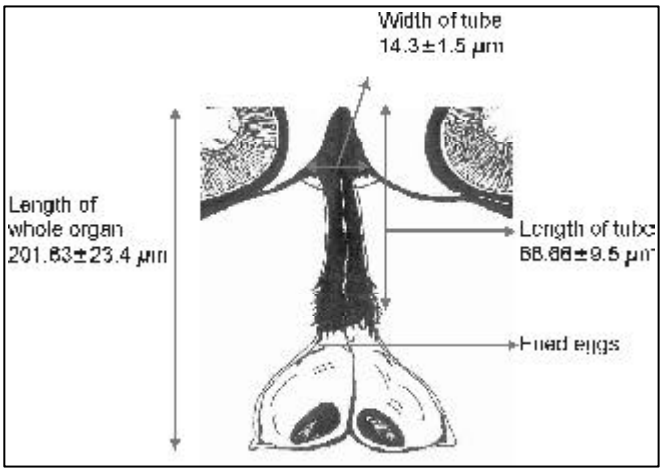

Fig. 2: Drawing of internal organ of male genitalia located in the 9th segment of abdomen (scale x 40 = 2.2 micron)

wildly separated, depending on the turgidity of the segment. Length, width and whole length of the organs were measured as 66.66 ± 9.5, 14.3 ± 1.5 and 201.63 ± 23.4 µm respectively in male (Table 1). Length, width and whole length of the organs in female were measured as 37.95± 4.0, 16.72 ± 1.4 and 219.34 ± 17.1 µm respectively (Table 1) and (Figs. 3 & 4).

Details of a method that refined for accurately determining the sex of alive IV instar larvae of *Aedes aegypti* L.[11] and that has been used successfully by others[12,13]. Establishing of our method using specific Neobauer slide is able to distinguish easily male from female at the larval stage. It is assumed that this is the first attempt to differentiate the anopheline larvae in the country and may be in the world.

Table 1. The special organ' s measurement [Mean ± SE (µm)] of male and female of anopheline larvae (n = 20)

Sex	Length of tube	Length of whole organ	Width of tube
Male	66.66 ± 9.5	201.63 ± 23.4	14.30 ± 1.5
Female	37.95 ± 4.0	219.34 ± 17.1	16.72 ± 1.4

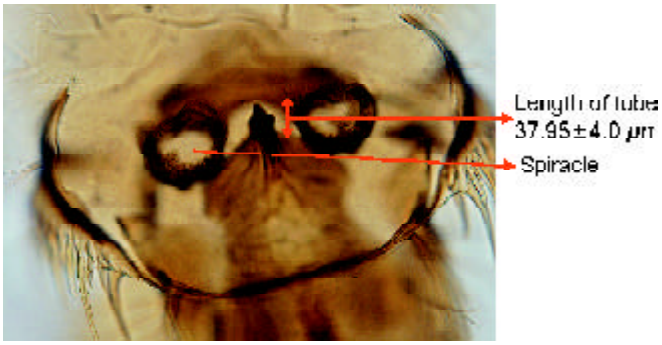

Fig. 3: Internal organ of female genitalia located in the 9th segment of abdomen which is parallel to spiracle of anopheline larvae

Studies of mosquito development must often determine the sex of preimaginal stages. Although female and male *Anopheles* larvae are similar in size at the beginning of the III instar, females subsequently grow more rapidly than the males when they are well-nourished. By the middle of the IV instar, the sexes can be accurately distinguished on the basis of size. If they are sub-optimally nourished, however, the size differences of female and male larvae and pupae are not as pronounced. Some protocols allow the larva to be reared to the pupa stage where female and male are easily distinguished on the basis of external structure: the cerci and 9th sternite of the female and the gonocoxites of the male[14]. Other protocols, however, require larva to be killed for analysis.

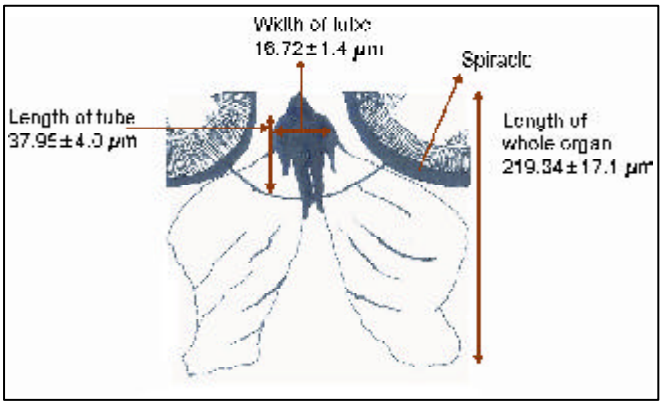

Fig. 4: Drawing of internal organs of female genitalia located in the 9th segment of abdomen (scale x40 = 2.2 micron)

Until now it has been virtually impossible to identify the sex of mosquito larvae, meaning it was impractical to separate the sexes and sterilise only males. Scientists have successfully controlled pest insects by releasing large numbers of both sexes that have been sterilized by irradiation or chemosterilants, to mate with the wild population. For eradication programmes, it required money and resources for rearing, irradiating and releasing females. Because the females of many insect pest species especially mosquito, mate only once, this method reduces the number of offspring. But, unlike male mosquitoes which feed on plant juices only, sterile females can still transmit malaria when they feed on human blood. So, to control malaria, only sterile males should be released. Using this method also means that pesticides with hazardous effect on environment are unnecessary. This method at the moment efficiently could separate male from female anopheline larvae manually but could be improved to an automated sorting machine.

This advance could have enormous implications for controlling mosquito populations. Scientists will be able to identify and separate different mosquito sexes much more easily. By identifying males and females at an early stage it will be possible to release sterile males into the population without the risk of releasing additional females. The release of sterile males has proven effective in controlling several insect pests when methods for sorting sex are available. Female mosquitoes are responsible for spreading malaria. By forcing females to breed with sterile males, we can prevent additional mosquito population. Although there have been a number of control programmes to eradicate malaria, none of these has been entirely successful, and many have also had side effects, such as environmental damage through insecticides. This advance could one day make a major impact on the burden of ill health caused by malaria. This work for the first time could be used as a control method in mosquito populations.

Acknowledgment

This work has received financial support from the Institute of Public Health Research, Academic Pivot for Education and Research, Medical Sciences/University of Tehran (Project No. 240/1594).

References

1. Saul SH. A genetic sexing system to improve the sterile insect technique against the Mediterranean fruit fly. *J Hered* 1990; *81*(1): 75–8.

2. Baker RH, Sakai RK, Raana K. Genetic sexing for a mosquito sterile-male release. *J Hered* 1981; *72*(3): 216–8.

3. Catteruccia F, Benton JP, Crisanti A. An *Anopheles* transgenic sexing strain for vector control. *Nat Biotechnol* 2005; *23*(11): 1414–7.

4. Seawright JA, Kaiser PE, Dame DA, Lofgren CS. Genetic method for the preferential elimination of females of *Anopheles albimanus*. *Science* 1978; *200:* 1303–4.

5. Bailey DL, Lowe RE, Dame DA, Seawright JA. Mass rearing the genetically altered MACHO strain of *Anopheles albimanus* Wiedemann. *Am J Trop Med Hyg* 1980; *29*(1): 141–9.

6. Robinson AS. Mutations and their use in insect control. *Mutat Res* 2002; *511*(2): 113–32.

7. Shearman DC. The evolution of sexdetermination systems in dipteran insects other than *Drosophila*. *Genetica* 2002; *116*(1): 25–43.

8. Robinson AS, Pham VL. Cytological linkage and insecticide studies on a genetic sexing line in *Anopheles stephensi* Liston. *J Hered* 1987; *58* (Pt 1): 95–101.

9. Kim SS, Seawright JA, Kaiser PE. A genetic sexing strain of *Anopheles quadrimaculatus* species A. *J Am Mosq Control Assoc* 1987; *3*(1): 50–3.

10. Shahgodian ER. A key to the anophelines of Iran. *Acta Med Iran* 1960; *3:* 38–48.

11. Chambers GM. A method for determining the sex of larval *Aedes aegypti* mosquitoes. *J Vector Ecology* 2005; *30:* 342–3.

12. Timmermann SE, Briegel H. Larval growth and biosynthesis of reserves in mosquitoes. *J Insect Physiol* 1999; *45:* 461–70.

13. Chambers GM, Klowden MJ. Correlation of nutritional reserves with a critical weight for pupation in larval *Aedes aegypti* mosquitoes. *J Am Mosq Control Assoc* 1990; *6:* 394– 9.

14. Christophers SR. *Aedes aegypti* (L.) the yellow fever mosquito. Its life history, bionomics and structure. Cambridge University Press, Cambridge. 1960.

Corresponding author: Dr. H. Vatandoost, Associate Professor, Department of Medical Entomology and Vector Control, School of Public Health & Institute of Health Research, Medical Sciences/Tehran University, P.O. Box. 6446, Tehran–14155, Iran.
E-mail: hvatandoost@yahoo.com

Received: 23 February 2007 *Accepted in revised form:* 28 May 2007