

**THE TOXICITY OF NEOSTIGMINE AGAINST
AEDES AEGYPTI LARVAE**

by

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requirements for the degree of Bachelor of Health
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CERTIFICATE

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ABSTRACT

The yellow fever, dengue fever and chikungunya are the important mosquito-borne disease to human in the world. *Aedes* mosquitoes have been widely studied for its role as the vector for these diseases. Various larvicides have been introduced to control the mosquito population. Among all, temephos is the most popular and widely used. The frequent use of temephos has resulted in the development of resistance in field populations of *Aedes* mosquitoes in Malaysia. Thus, this study was carried out to explore the potency of utilizing Neostigmine Injection B.P. 2.5 mg (neostigmine) as an alternative larvicide subsequently delaying the development of resistance of mosquitoes against temephos. The potency was evaluated by assessing the toxicity of neostigmine against larvae of *Ae. aegypti*. Neostigmine is a drug used in the treatment for myasthenia gravis due to its effects as an anticholinesterase. The drug was chosen because of its mode of action, which is similar to organophosphate larvicide. In the study, four different dosages of neostigmine were evaluated, i.e. 12.5, 25, 50 and 100 ppm against the late third to early fourth instar larvae of *Ae. aegypti* using the modified WHO (1981) method. Temephos was included in the test regime as the benchmark and distilled water only as the control. The tests were conducted in five replicates for twenty-one days. The percentage of cumulative mortality of larvae was calculated and analyzed with Independent T test. The results showed 100 ppm of neostigmine has the highest percentage of mortality (99.2 %), followed by 50 (97.6 %), 12.5 (96.8 %) and 25 (94.4 %) ppm. The increase of mortality was significant ($P < 0.05$) for neostigmine at 100 ppm dosage compare to control. For treatment of 50 ppm of neostigmine, the increase of mortality was only significant at $P < 0.10$. However, when compared to temephos, the percentage of mortality generated by 100 ppm neostigmine was significantly ($P < 0.05$) lower.

Ketoksikan *Neostigmine* Terhadap Larva *Aedes aegypti*

ABSTRAK

Demam kuning, demam denggi dan *chikungunya* merupakan antara penyakit bawaan nyamuk yang penting bagi manusia. Nyamuk *Aedes* telah pun luas dikaji dalam peranannya sebagai vektor. Pelbagai jenis racun larva telah diperkenalkan bagi mengawal populasi nyamuk dan di antaranya *temephos* merupakan racun yang paling luas digunakan. Kekerapan penggunaan *temephos* yang tinggi telah menyebabkan kerintangan terhadap *temephos* di kalangan populasi nyamuk *Aedes* di Malaysia. Oleh itu, kajian ini telah dijalankan bagi menguji potensi ubat Neostigmine Injection B.P. 2.5 mg (*neostigmine*) sebagai alternatif kepada racun larva dan seterusnya memperlahankan kerintangan larva terhadap *temephos*. Potensi ubat *neostigmine* dikaji dengan memerhatikan ketoksikannya terhadap larva *Ae. aegypti*. Ubat *neostigmine* mempunyai kesan sebagai *anticholinesterase* dan digunakan untuk merawat penyakit seperti *myasthenia gravis*. Ubat ini dipilih kerana ia mempunyai tindakan yang mimik racun serangga kumpulan organofosfat. Dalam kajian ini, empat kepekatan *neostigmine* yang berlainan telah digunakan, iaitu 12.5, 25, 50 dan 100 ppm terhadap larva peringkat lewat instar ketiga dan peringkat awal instar keempat dengan menggunakan kaedah WHO (1981) yang telah diubahsuai. *Temephos* turut dirangkumi dalam set ujian sebagai penanda aras manakala air suling digunakan sebagai kawalan. Kajian adalah dijalankan dalam lima replikasi bagi dua puluh satu hari. Peratusan kematian terkumpul larva dikira dan dianalisa dengan menggunakan ujian *Independent T*. Keputusan kajian menunjukkan *neostigmine* pada kepekatan 100 ppm menunjukkan peratusan kematian larva yang paling tinggi (99.2 %). Ini diikuti dengan kepekatan 50 (97.6 %), 12.5 (96.8 %) dan akhirnya 25 (94.4 %) ppm. Peningkatan peratusan kematian adalah signifikan (*P*

< 0.05) bagi kepekatan *neostigmine* pada 100 ppm berbanding dengan kawalan. Manakala bagi *neostigmine* 50 ppm, peningkatan peratusan kematian hanya signifikan pada $P < 0.10$. Walau bagaimanapun, apabila perbandingan dibuat dengan *temephos*, peratusan kematian disebabkan oleh 100 ppm *neostigmine* adalah lebih rendah ($P < 0.05$).

1.0 INTRODUCTION

World Health Organization (WHO) classified the virus for dengue fever as the most important arbovirus to human in the world. *Aedes aegypti* has been found to transmit the dengue viruses. It is estimated that each year, a total of 50 million infections of dengue virus occur with 500,000 cases of dengue hemorrhagic fever and 12,000 deaths (Academy of Medicine Malaysia, 2003). The factors that contribute to dengue outbreaks are increase of uncontrolled demographic, poor urban planning, reduced epidemiological surveillance and progressive resistance of the vector mosquito to insecticides (Carolina *et al.*, 2004). Besides the transmission of dengue viruses, *Aedes aegypti* is also the vector of the viruses of yellow fever and chikungunya.

Integrated Mosquito Control program is carried out in countries facing the treat in order to suppress mosquito populations. Mosquito control program targets the larval stage of *Ae. aegypti* to ensure effective protection to human population. According to Dharmagadda *et al.*, (2005), it is much easier to control the mosquito population in the larval stage and this can reduce the overall application of adulticides.

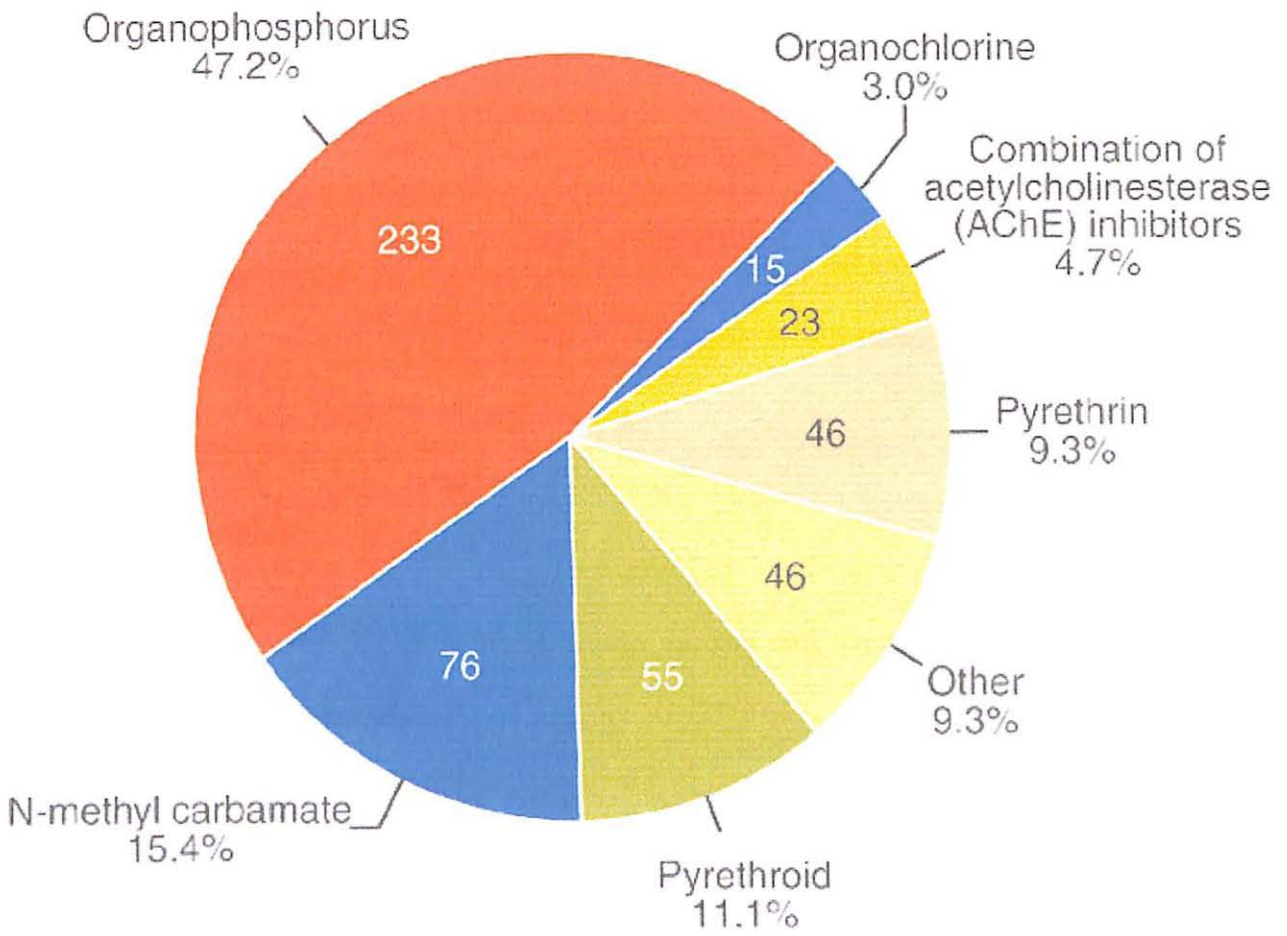
There are various examples of larvicides, like peppermint oil (Ansari *et al.*, 2000), *Bacillus thuringiensis israelensis* (bti), temephos, methoprene and monomolecular films (EPA, 2002). Temephos is a popular larvicide. Temephos is an organophosphate pesticide that affects the nervous system by reducing the ability of cholinesterase to function properly in regulating acetylcholine (Lee *et al.*, 1997). If acetylcholine is not properly controlled by cholinesterase, the nerve impulses or neurons remain active longer than they should, which continuously stimulates the nerves and muscles.

Subsequently causing symptoms such as weakness or paralysis of the muscles. Due to the frequent use of temephos, it has resulted in the development of resistance in field populations of *Aedes* mosquitoes in Malaysia (Seleena *et al.*, 2001). Besides the resistance, temephos and other organophosphate could cause contamination of water and injury to human or animals that were not the targets of the pesticide. Figure 1.1 shows the distribution and number of organophosphate-related illnesses in year 1998 to 1999 in United States (NIOSH, 2004).

Neostigmine is an anticholinesterase drug approved by Ministry of Health, Malaysia for use in treatment of myasthenia gravis, postoperative distension or urinary retention and antidote for tubocurarine (Delmar, 2002). The effect of neostigmine on neural system is similar to organophosphate insecticides, such as temephos, that is as an acetylcholinesterase inhibitor (James *et al.*, 1996, Delmar, 2002). Thus, it is believed that neostigmine could also act as a larvicide.

In addition, risk of the health hazard from neostigmine could be relatively lower than temephos based on the use of neostigmine as drug for human compared to temephos. Thus, the use of neostigmine as larvicide may seem to provide a safer option compared to temephos.

The objective of this research was to explore the potency of neostigmine as larvicide by evaluating the toxicity of neostigmine against larvae of *Ae. aegypti*. If the result is positive, then it indicates the potency of neostigmine to be used as an alternative to temephos subsequently delaying the development of resistance of mosquitoes against temephos.



(NIOSH, 2004)

Figure 1.1: Distribution and number of pesticide-related illnesses by insecticide chemical class in year 1998 to 1999 in United States

2.0 LITERATURE REVIEW

2.1 Dengue

WHO reported dengue as the most common mosquito-borne viral disease of humans. In recent years, it became a major international health concern, as 2.5 billion people live in areas where dengue viruses can be transmitted globally. Dengue fever (DF) has become an important public health problem as the number of reported cases continues to increase, especially the severe forms of the disease, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), or with unusual manifestations such as central nervous system involvement (Wej *et al.*, 2004).

The geographical spread of both the mosquito vectors and the viruses has led to the global resurgence of epidemic DF and emergence of DHF in the past 25 years with the development of hyperendemicity in many urban areas in the tropics. The geographical spread, incidence and severity of DF and DHF are increasing in the America, South-East Asia, the Eastern Mediterranean and the Western Pacific. It is estimated that each year 50 million infections occur, with 500,000 cases of DHF and at least 12,000 deaths (Academy of Medicine Malaysia, 2003). Figure 2.1 shows distribution of *Aedes* and dengue fever in the year of 2003.

World Distribution of Dengue - 2003

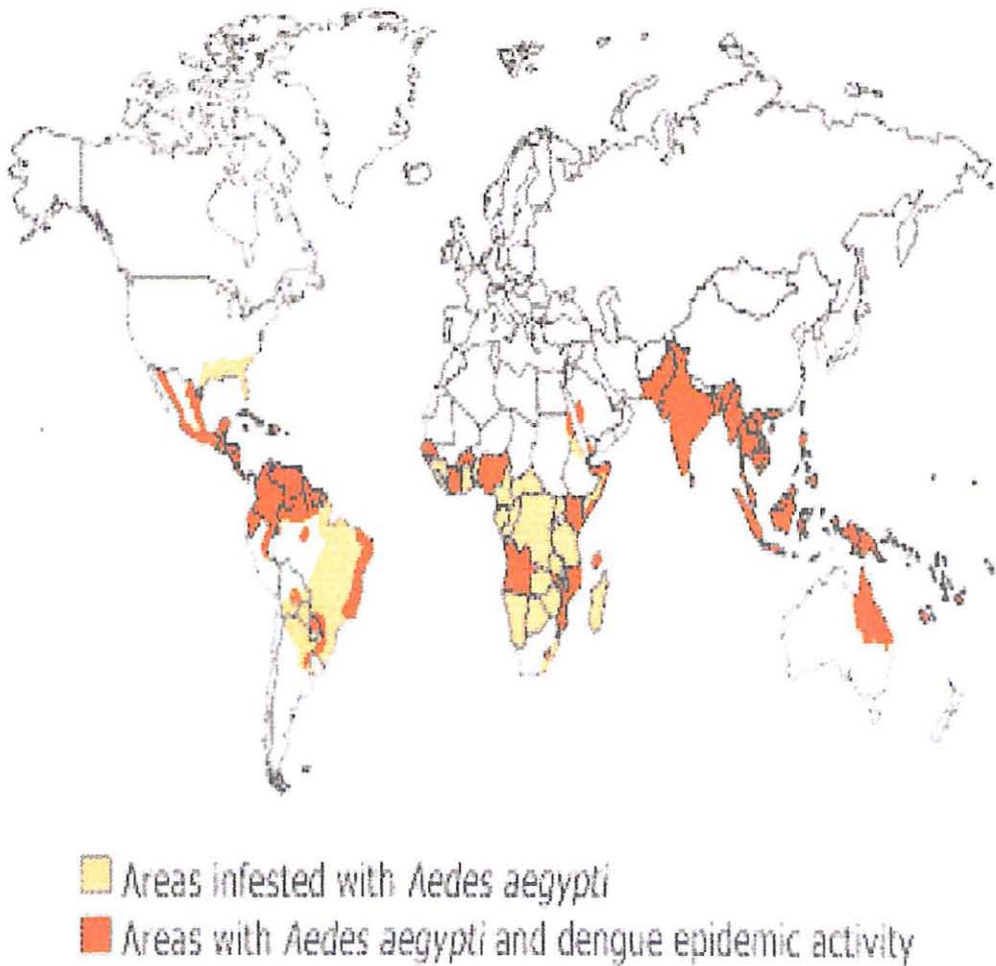
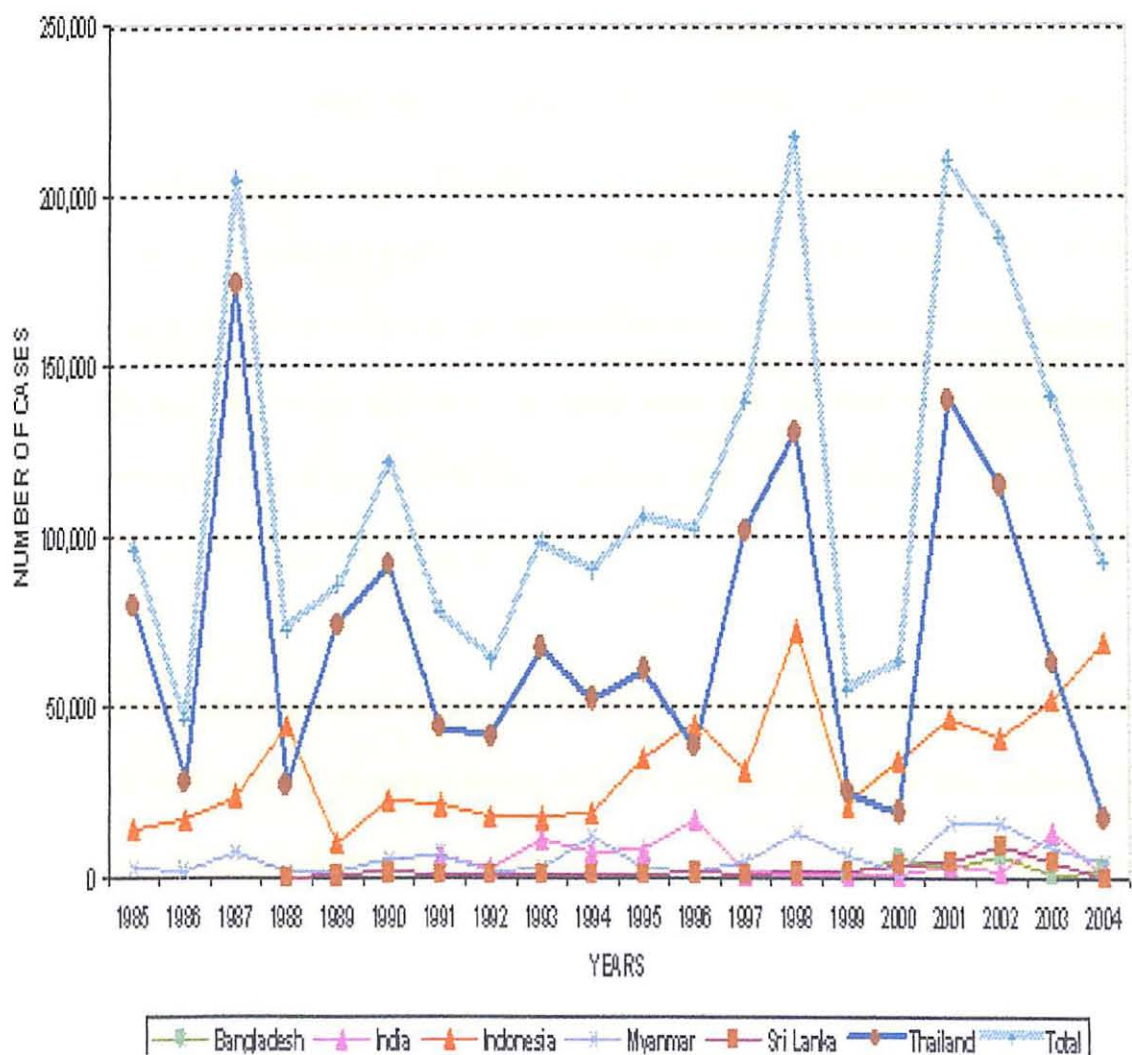


Figure 2.1: The distribution of *Aedes* and dengue fever in the year of 2003 (CDC, 2006)

The earliest report of a dengue epidemic in Peninsular Malaysia was from Singapore and it was in 1901. In Malaysia, the presence of dengue fever was first reported in 1902 in Penang Island (Rebecca, 1992). The first major outbreak of DHF in Malaysia occurred in the year 1973 and dengue type 3 was considered to be the main causal type (Wallace *et al.*, 1980).

In the year 2003, about 140,000 cases of DF and DHF were reported in the South-East-Asian region (Figure 2.2), while the case fatality rate for the same year is 0.85 % (WHO, 2004). In Malaysia, 16,368 cases were reported in the year 2001 and 22 % of these were children aged 14 years and below. Similarly, the case fatality rate for DHF is high, ranging from 5 % to 6 % per annum for both children and adults. In that particular year, there were 50 cases of death from DF and the incidence rate was 68.79 per 100000 (Academy of Medicine Malaysia, 2003).

According to Carolina *et al.*, (2004), the factors accounting for dengue outbreaks are increase of uncontrolled demographic, poor urban planning, reduced epidemiological surveillance and progressive resistance of the vector mosquito to several insecticides produced by the chemical industry.



* Data up to August 2004

Figure 2.2: Reported Cases of DF/DHF in Selected Countries in SEA Region from 1985 to 2004* (WHO, 2004)

2.2 *Aedes aegypti* mosquitoes

Mosquitoes are the most prominent of the numerous species of blood-sucking arthropods that irritate man and other warm-blooded animals (Maurice and Robert, 1969). They are also the vectors that are responsible for spreading serious human diseases like malaria, Japanese encephalitis, West Nile virus, yellow fever, dengue and filariasis (Veena *et al.*, 2005, Deborah *et al.*, 2004). Mosquito-borne diseases have emerged or re-emerged as significant human health problems due to a number of factors, which include lack of progress in vaccine development, emergence of drug resistance in pathogens and insecticide resistance in mosquitoes, and the decline in socioeconomic conditions in many disease endemic countries that limits disease monitoring and mosquito control efforts (David *et al.*, 2004).

The mosquito species, *Aedes aegypti*, is the primary, worldwide arthropod vector of the viruses of yellow fever, dengue viruses and chikungunya (Maurice and Robert, 1969, Nongkran *et al.*, 2005, David *et al.*, 2004).

The taxonomy of the mosquito is as stated below:

- Kingdom: Animalia
- Phylum: Arthropoda
- Class: Insecta
- Order: Diptera
- Family: Culicidae
- Genus: *Aedes*
- Species: *Aedes aegypti*

According to David *et al.*, (2004), this species of mosquito is considered as the most tractable mosquito species for laboratory culture, and has been used for detailed laboratory investigations of mosquito biology including morphology, physiology, genetics, vector competence, and recently, molecular evolution applications.

2.3 Geographic distribution

Canyon (2001) in his review article mentioned that while the latitudinal limits of *Ae. aegypti* are 45° North and 40° South of the equator, *Ae. aegypti*'s distribution is more closely correlated with the 10 °C isotherms, and is found throughout most tropical to subtropical regions of the world (Womack, 1993). Canyon also pointed out that the estimation of *Ae. aegypti* distribution and density are affected by the life-limiting factors of latitude, altitude, temperature, rainfall, humidity, season, habitat and dispersal.

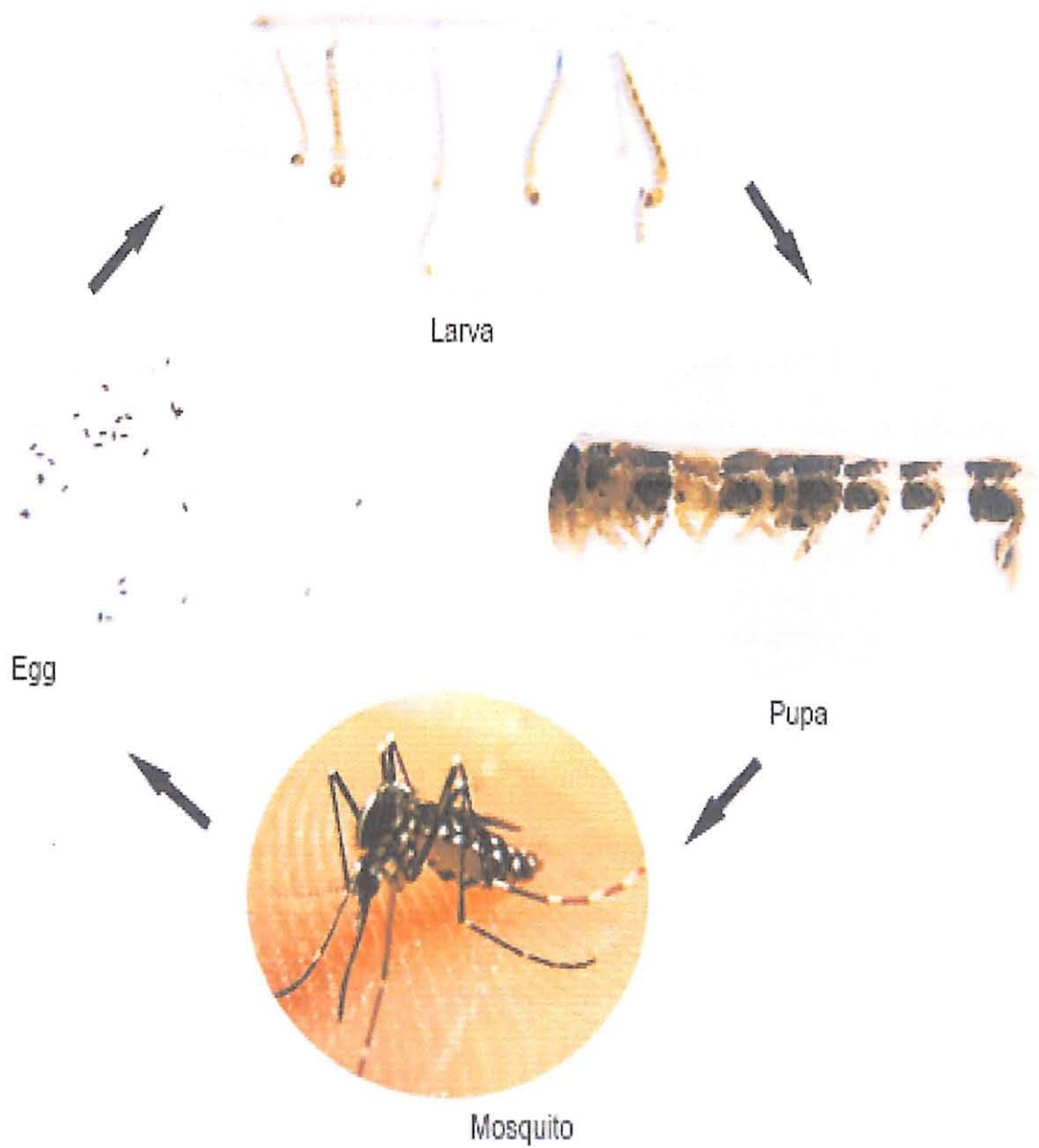
Ae. aegypti is highly susceptible to temperature variations and its survival is poor in hot and dry climates (Womack, 1993). According to Maurice and Robert (1969), it soon dies in the open air at a temperature of 7 to 8 °C, succumbing in a few seconds when exposed to a temperature of 0 °C. It also dies quickly at 37 °C (Maurice and Robert, 1969).

2.4 Life cycle of *Ae. aegypti*

Metamorphosis is the series of changes in the organism to achieve the size and development of the parent, in which the young insect undergoes greater or less change in size, form and structure. Among all types of metamorphosis, *Ae. aegypti* undergoes complete metamorphosis or holometalobous development (Figure 2.3).

During the intervening metamorphosis, this insect undergoes considerable structural and functional remodeling. The larval stage is an active, feeding and wormlike. Wing development is suppressed. The larva increases in size through successive instars, and at the end of the larval period, it passes into pupal stage, in which a radical transformation takes place (Robert *et al.*, 1991).

Many internal larval tissues are destroyed by autolysis and phagocytosis, and adult structures develop from imaginal cells that are set aside during embryonic and larval life. At pupal stage, the internal remodeling occurs within an epidermal body wall that changes from a relatively simple segmented tube to the geometrically complex triad of head, thorax, and abdomen characteristic of adult insects (Gema *et al.*, 2003). According to James *et al.*, (2005), the transformation of the immature insect into reproducible adult is under a cascade of transcriptional events that occur in response to hormonal signals. It is dependent upon the balance of both ecdysone and juvenile hormone titers.



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Figure 2.3: The life cycle of *Aedes aegypti* mosquito (National Environment Agency, 2005)

2.4.1 Adult

Ae. aegypti is small in comparison to others. It is usually between three to four millimeters in length, discounting leg length. The body is composed of a well-defined head, thorax and abdomen. It is marked with silvery-white or yellowish white bands and stripes on a nearly black background (Roland *et al.*, 1998). It has a “lyrelike” pattern dorsally on its thorax formed by two outer-curved yellowish-white lines and two median parallel lines. The legs are seen as banded, and the last segment of the hind leg is entirely white. Its head is covered with broad flat scales with a single row of upright forked scales (Maurice and Robert, 1969).

The head is small and almost spherical. It is joined to the thorax by a narrow membranous connection. A large pair of compound eyes occupies the lateral surface of the head. This mosquito has a pair of long, filamentous and segmented antennae. The male mosquito’s antennae are conspicuous and plumose while the female are pilose. The proboscis is long and slender, and projects downwards and forwards from the lower front margin of the head. The proboscis of *Ae. aegypti* consists of a long, "u"-shaped, fleshy, sheath-like structure, namely labium, enclosing a group of six stylets and is adapted for piercing and sucking. The labium encloses paired maxillae, mandibles, an epipharynx and a hypopharynx. The roof of the proboscis is formed by the labrum, a shelf like projection of the head. The hypopharynx carries a salivary duct, through which an anticoagulant is projected from the floor of the oral cavity, and delivered into the hosts tissues (Suzanne, 2004).

Culicidae mosquitos’ families have three pairs of long legs and two wings attached to the thorax. The wings are covered with dark scale. The sides of the thorax of Culicidae

may be covered with scale bristles and bear several groups of hair or bristles, but *Aedes* species do not have spiracular bristles in front of the mesothoracic spiracles. Instead, *Aedes* species have post-spiracular bristles (a group of bristles immediately behind the mesothoracic spiracle) (Suzanne, 2004). The hind legs have pale scales on the basal three-quarters of the femur, with dark scales covering dorsally on the apical two-thirds and ventrally on the apical third. The tibia is dark but tarsi with pale basal bands on 1- 4 and 5 are all pale (Russell, 1996).

The abdomen is elongated and nearly cylindrical. It consists of ten segments, of which only eight are readily available as the ninth and tenth segments are greatly modified for sexual functions. Gonopore, the external opening of the reproductive organs, is situated on the posterior portion of abdomen. They have a pointed tip to the abdomen with the paired cerci (jointed appendage at the tip of the abdomen in insects) protruding. The female *Aedes* mosquitoes' abdomens are tapered apically, with the eighth segment withdrawn into the seventh (Suzanne, 2004).

Ae. aegypti is highly anthropophilic, although it may feed on other available warm blooded animals. Being a diurnal species, females have two periods of biting activity, one in the morning for several hours after daybreak and the other in the afternoon for several hours before dark (WHO, 1999). *Ae. aegypti* may feed on more than one person when there is an interruption during feeding (WHO, 1999). The adult male (and occasionally the females) feed on nectar and other plant juices (Suzanne, 2004). *Ae. aegypti* prefers to rest in dark, humid, secluded places inside houses or buildings. Less often, it can be found outdoors in vegetation or other protected sites (WHO, 1999).