DISSERTATION

submitted to the

Combined Faculties for the Natural Sciences and for Mathematics

of the Ruperto-Carola University of Heidelberg, Germany

for the degree of

Doctor of Natural Sciences

presented by

Mila Mahilum, Master of Science in Biology

Cebu City, Philippines

Oral examination: February, 2003

Biochemical, molecular and microscopic studies on mosquitoes with special emphasis on *Wolbachia* infections, diagnostic assessment of Dengue viruses and biological control of Dengue vectors

Referees: Prof. Dr. Volker Storch
Prof. Dr. Elisabeth Pollerberg

ACKNOWLEDGMENT

Heartfelt thanks and gratitude to: Prof. Dr. Volker Storch, Head of the Zoological Institute, University of Heidelberg, for acting as adviser and first referee of the Ph. D. work, for sharing his expertise concerning the techniques on electron microscopic study and most especially for the guidance and help in making this work possible.

Dr. Norbert Becker, Scientific Director of the German Mosquito Control Association (KABS)/GFS, for acting also as adviser of the Ph. D. work, for the guidance and support in the course of the studies and most especially for sharing his valuable knowledge especially in regards to the biology and control of the mosquitoes.

Prof. Dr. Elisabeth Pollerberg, Zoology Institute, University of Heidelberg, for the help and being the second referee of the Ph. D. work.

Dr. Susanne Lankenau and Dr. Dirk Lankenau, Zoological Institute, University of Heidelberg, for the support of the *Wolbachia* study. Mrs. Renate Mummert, Mrs. Claudia Kempendorf and Mrs. Gisela Adam, Zoology Institute, University of Heidelberg, for the assistance on electron microscopic study and Mrs. Gabriele van der Linden for the technical assistance.

Mr. Thomas Weitzel and Mr. Artur Jöst for teaching the techinque on enzyme electrophoresis as well as for the continuous support of the biochemical study. Mr. Heiko Kotter and Mr. Mattias Beck for helping in the collection of mosquito larvae. Ms. Silvia Guerreri for assisting the Elisa study, Ms. Regina Teo for reviewing some parts of the manuscript, Dr. Beate Heeger and Dr. Thomas Heeger, Dr. Filipina Sotto, Ms. Agnes Tomayao, Ms. Abigail Genelsa, Ms. Pia Ternes, and Mr. Markus Ulrich for the help of the study. Dr. Paul Schädler, President of the German Mosquito Control Association (KABS) and Gesellschaft zur Förderung der Stechmückenbekämpfung (GFS) for the support of the study.

To all the people who spared assistance whom I may not have mentioned above, to all my beloved friends, my family for giving me the inspiration. Above all, the author is most grateful and thankful to the German Academic Exchange Program (DAAD) for making this work possible and most especially for the financial support during the whole period of the Ph. D. work.

CONTENT

1. GENERAL INTRODUCTION

1.1. General background of Culicidae

Insects are the largest and most successful group of arthropods (Scott, 1980). Reasons for their success is attributed to their high rate of reproduction, their ability to adapt to almost every environmental condition and lastly, their accessibility to a variety of food sources. Many studies on insects are focused on such different research fields such as genetics, evolutionary biology, systematics, ecology and applied zoology because of their great medical and agricultural importance. Due to their advantageous characteristics in the laboratory, some species are good experimental animals (e.g. *Drosophila melanogaster* Meigen) and also serve as model organisms in various researches.

Insects and humans affect each other in many ways. Since both are very successful species, they sometimes affect each other in a negative way. Insects are considered by most people to be annoying. They bite or sting and can infect humans and other tetrapods with diseases. The most important vectors of diseases are the mosquitoes. For this reason, they are certainly among the best known family of insects that have of great importance in research. Their systematics have been studied not only at the traditional morphological level but also utilizing, at least in some groups, crossing experiments, cytological and molecular genetic analysis (Bullini & Coluzzi,1978).

Mosquitoes are placed in the Family Culicidae, suborder Nematocera of the order Diptera (the two-winged flies or true flies). The Culicidae contain over 3,200 species and are divided into 3 subfamilies: Anophelinae, Culicinae, and Toxorhynchitinae (Scott, 1980). They are one of the more primitive families of Diptera being more closely related to midges, gnats and crane flies. They occur throughout the world except in places that are permanently frozen (Clements, 1992). Mosquitoes are widely investigated by the researchers for the reason that they are the hosts to a variety of pathogens and parasites including viruses, bacteria, protozoans and nematodes. Thus, by their blood-sucking habit, they serve as agents of human and animal diseases.

1.2. The biology of Culicidae

Like all other Dipterans, mosquitoes exhibit complete metamorphosis. They need aquatic habitats for their development. After hatching they pass through four larval instars and a pupal stage where the transformation to the adult stage takes place. Most species are unautogenous, that means after copulation the females have to take a blood meal to complete egg development. Only a few species of mosquitoes are autogenous, they develop first egg batches without a blood-meal (e.g. *Cx. p. pipiens* biotype *molestus*) (Becker et al., 2003).

1.2.1 Oviposition

Female mosquitoes are capable of laying between 50 to almost 500 eggs, two to four days or even more in cool temperate climates after having a blood meal. Mosquitoes can be divided into two groups depending on their egg laying behaviour and whether or not the embryos enter into a period of dormancy (externally triggered resting period) or diapause "genetically fixed resting period" (Barr, 1958).

In the first group, females deposit the eggs onto the surface of the water either singly (e.g. *Anopheles*) or in egg batches (e.g. *Culex*). To cite an example, females of *Culex* lay their eggs in rafts which are comprised of several hundred eggs locked together in a boat-shaped structure. Anophelines lay eggs singly while standing on the water or hovering over it. The eggs of this mosquito group are adapted for floating and can easily be destroyed by dessication. The embryos do not enter a dormancy or diapause and hatch when the embryonic development is completed. Species having these non-dormant eggs usually produce several generations each year. Their developing stages are found for the most part in more permanent waters where one generation succeeds another during the breeding season. The number of generations depends on the length of the breeding season as well as abiotic and biotic conditions, most especially the temperature which influences the speed of the development.

The factors or conditions that lead to the choice of a breeding site by the females in laying their eggs onto the water surface are still unknown for many species. Factors such as water quality, light intensity, availability of food, and local vegetation, are some of the conclusive factors for the choice of a breeding site.

The second group of female mosquitoes lays eggs which do not hatch immediately after oviposition. The most interesting is the egg-laying behaviour of the floodwater mosquitoes (*Aedes vexans*). The eggs are laid into small depressions or between particles of moss with a high degree of soil moisture in order to protect the sensitive eggs from drying out during embryogenesis (Barr & Azawi, 1958; Horsfall et al., 1973).

1.2.2. Embryonic development

The embryonic development of mosquitoes starts almost immediately after the eggs have been laid. It takes about two days to a week or more depending on the temperature until the embryos are fully developed .

The course of embryonic development reflects also a special adaptation to various abiotic conditions in the larval habitat (Becker, 1989a). The non-dormant eggs of *Culex* hatch shortly after the embryonic development is completed. The length of time required is dependent almost entirely on the temperature. The embryonic development of *Aedes* species usually takes longer. For instance, larvae of *Ae. vexans* are ready to hatch four or eight days after oviposition, when the eggs are kept at 25°C and 20°C, respectively (Horsfall et al., 1973; Becker, 1989a). This means that the embryonic development of *Culex pipiens* usually takes only half as long as that of *Ae. vexans* which assures a quicker generation renewal of the former.

1.2.3. Hatching

Aedes mosquitoes have developed a high sophisticated mechanism which regulates the hatching process as a direct adaptation to the greatly fluctuating abiotic conditions existing in the temporary waters where these mosquitoes breed (Gillett, 1955; Telford, 1963; Horsfall et al., 1973; Beach, 1978; Becker, 1989b; Becker et al., 2003).

Temperature fluctuations, varying degrees of moisture in the air and soil as well as changes in the day-length are the factors that are most likely to have an influence on diapause or on hatching inhibition and readiness (Brust & Costello, 1969).

The hatching process has been extensively investigated in *Ae. vexans* eggs (Becker, 1989a; Horsfall et al., 1973). After the content of oxygen decreases in the water of the breeding site, the larvae initiates the shell rupture by pressing the so-called "egg tooth", an egg burster located posterio-dorsally on the head capsules of the larva, onto the egg shell. As a result the shell splits along a particular line at the anterior end of the egg. A cap (anterior part of the egg shell) comes away and the larvae escape by swallowing water into the gut which forces the body from the shell (Clements, 1992). The whole process of hatching takes only a few minutes.

1.2.4. Larvae

The larva is divided into three distinct parts: a) the head with mouthparts, eyes and antennae; b) the broader thorax and c) the abdomen which is composed of seven almost identical segments and three modified posterior segments. The posterior segments bear 4 anal papillae which function in regulating electrolyte levels. At the VIII abdominal segment, a siphon in culicines, or only spiracular lobes in anophelines, are developed where the tracheal trunks open at spiracles for the intake of oxygen. Usually the culicine larvae hang their head downwards from the water surface whereas, anopheline larvae lie horizontally at the water surface.

Food of the larvae consist of microorganisms, algae, protozoans, invertebrates and detritus. On the basis of their feeding behaviour they may be classified into filter or suspension feeders, browsers or predators. The filter feeders collect food particles which are suspended in the water column (*Culex*) or the microbial film at the air-water interface (*Anopheles*). The larvae of the filter feeders (*Culex*) typically hang on the water surface, filtering the water column beneath the surface by beating their head brushes (lateral palatal brushes) towards the preoral cavity. This generates water currents which carry food particles towards the mouth. Mosquito larvae are usually not selective with regards to the food they ingest. However, the size of the particles should be less than 50 μm. Larvae can move also slowly in the water column while filtering the food. The browsers (mostly *Aedes*) collect food by re-suspension, scraping or shredding particles, microorganisms, algae, and protozoa from the surface of submerged substrates or the microbial film at the air-water interface (*Anopheles*). Even small parts of dead invertebrates and plants can be bitten off with the mouth parts (Becker et al., 2003).

In contrary, the anopheline larva hangs horizontally under the water surface with its dorsal side uppermost and the mouthparts directed downwards. Upon feeding, the larva rotates its head through 180° and creates a water current by beating its head brushes to collect the food organisms in the surface film.

Disturbances of the water surface cause the larvae to dive for a short period of time. They dive by flexing the abdomen and moving backwards. When the larvae return to the water surface, they swim backwards until the abdomen comes into contact with the surface (Becker et al., 2003). Larvae moult four times at intervals before reaching the pupal stage. At each moult the head capsule is increased to full size which is a characteristic of the next instar, whereas the body grows continuously. Each moult is coordinated by the relative concentrations and interactions of juvenile hormone and ecdysone (a moulting hormone).

1.2.5. Pupae

Just like the larvae, pupae are also aquatic. The pupal stage usually lasts about two days. However, this period may be reduced or extended, at higher or lower temperatures, repectively. During the pupal stage the process of metamorphosis takes place. Some larval organs are histolized, while the body of the adult is formed through the development of imaginal discs (cells or groups of cells that stayed quiescent in the larval body until the pupal stage). In particular, the fat body of the larvae is transferred to the adult stage and used as a source of vitellogenines for autogenous egg formation or as a reserve for hibernation. Characteristically, the head and thorax of the pupa are fused into a prominent cephalothorax which carries anterio-laterally two respiratory trumpets. The abdomen which terminates with two paddles and is kept flexed under the cephalothorax. The trumpets are connected with the mesothoracic spiracles of the developing adult to provide the organism with oxygen. When at rest the pupae float motionless in the water surface. When the pupa is disturbed, it dives by straightening the abdomen and spreading the paddles, then it rapidly flexes the abdomen which has retained the larval musculature. In contrast to the larvae which have to swim actively to the water surface, the pupa floats passively back to the surface after diving. Mosquito pupae, unlike the pupae of most other insects, are quite mobile (Becker et al., 2003).

1.2.6. Emergence of adults

When the final stage of metamorphosis is completed, gas is forced between the pupa and the adult cuticle as well as into its midgut. The pupa straightens the abdomen into a horizontal position, and by swallowing air, it further increases the internal pressure. The cephalothoracic cuticle of the pupa splits along the ecdysial line and the adult slowly emerges from the pupal skin. The emerging adult moves carefully to avoid falling onto the water surface, while its appendages are still partly in the exuvia. In this phase the emerging individual is highly susceptible to strong winds.

After emergence the adult increases the haemolymph pressure which causes the legs and wings to stretch. It immediately ejects droplets of fluid to empty the gut, while air disappears from the gut for some hours later. Within a few minutes when the soft cuticle has sclerotized, it is able to fly (Gillet, 1983; Becker et al., 2003).

There is also a difference between male and female sexual maturity at the time of emergence. Male mosquitoes are not sexually mature at emergence. They have to rotate their hypopygium at 180° before they are ready to mate, which takes about one day. The males emerge 1-2 days before the females, in order to achieve sexual maturity at the same time as the females.

1.3. Medical importance of mosquitoes

Human population especially in developing countries is increasing continously. As this happens, breeding sites of mosquitoes like garbages and stagnant canals are also increasing. This situation has greatly affected the population of mosquitoes that carry diseases (WHO, 1997a).

Mosquitoes are responsible for the transmission of many medically important pathogens and parasites such as viruses, protozoans and nematodes that cause serious diseases like malaria, dengue, yellow fever, encephalitis or filariasis (Kettle, 1995; Beaty & Marquardt, 1996; Lehane, 1991). Transmission can be mechanical (e.g. myxoma virus causing myxomatosis in rabbits) or biological. The latter is more complex because it involves an obligatory period of replication and/or development of the pathogen or parasite in the vector insect. Due to their

blood-sucking behaviour, mosquitoes are able to acquire the pathogens or parasites from one vertebrate host and pass them to another, if the mosquito's ecology and physiology is appropriate for the transmission. Highly efficient vectors have to be closely associated with the hosts, and their longevity has to be sufficient to enable the pathogens/parasites to proliferate and/or to develop to the infective stages in the vector. For successful transmission, usually multiple blood-meals are necessary (Becker et al., 2003).

In terms of morbidity and mortality caused by vector-borne diseases, mosquitoes are the most dangerous insects confronting mankind. They threaten more than two billion people in tropical and subtropical regions, and have substantially influenced the development of mankind, not only socio-economically but also politically (Bruce-Chwatt & de Zulueta, 1980).

1.3.1. Malaria

Malaria is caused by the protozoans *Plasmodium* spp. and continues to be the most important vector-borne disease. It affects more than 100 tropical countries, with more than 40% of the world population are at risk. Some 300 million people are believed to be infected with malaria parasites, with 90% of them living in tropical Africa (WHO, 1993; WHO 1997a,b). In Africa, the disease is probably responsible for 500,000 to 1.2 million deaths annually, mainly among children below the age of five. The enormous total of lives and days of labour lost, the costs of treatment of patients, emphasize the negative socio-economic impact of the disease. The annual costs of malaria in Africa alone were estimated to be almost 2 billion US$ in 1995 (WHO, 1997a,b).

Four species of the genus *Plasmodium* (*P. falciparum, P. vivax, P. ovale* and *P. malariae*) cause human malaria and are transmitted solely by anopheline mosquitoes. The *Plasmodium* species have a complex replication and transmission cycle with the sexual replication in mosquitoes and the asexual replication in vertebrates.

In humans, malignant malaria caused by *P. falciparum* is the most severe form resulting in a life-threatening complications such as anaemia and cerebral malaria. This is a frequent cause of mortality among children and can kill up to 25% of non-immune adults within two weeks. This form of malaria is called malaria tropica and occurs mostly in tropical and subtropical

areas, being limited by a summer isotherm of 20°C which is necessary to complete sporogony of the parasite in the mosquito. In contrast, *P. vivax* can complete sporogony in mosquitoes in areas with a summer isotherm of 16°C (Wernsdorfer, 1980).

1.3.2. Virosis

Arboviruses (Arthropod-Borne-Viruses) are defined as viruses that replicate in arthropods and are transmitted by arthropods to vertebrates. The arthropod becomes infected by feeding on blood from an infected vertebrate during viremia (virus circulation in the peripheral blood vessels), and after proliferation in the vector, the virus can be transmitted to another vertebrate-host (horizontal transmission). Arboviruses can also be passed from one arthropod generation to another by transovarian transmission (vertical transmission). Thus, some of these viruses are known to be capable of overwintering in the egg stage of the vector (e.g. some *Aedes* species).

More than 300 arboviruses are listed by Francki et al. (1991) and more than 500 by Karabatsos (1985). Approximately 100 viruses can infect humans and 40 are able to infect livestock (Monath, 1998). The most important viruses transmitted by mosquitoes to humans or other vertebrates are found in three families: the Togaviridae with the genus *Alphavirus* (*e.g.* Sindbis virus, equine encephalitis viruses), Flaviviridae with the genus *Flavivirus* (e.g. yellow fever virus, dengue 1-4 viruses, West Nile virus, Japanese and St. Louis encephalitis/SLE-viruses) and the Bunyaviridae with the genera *Bunyavirus* (e.g. California Group), and *Phlebovirus* (Rift Valley fever) (Murphy et al., 1995). Human arboviral diseases are classified by the major clinical symptoms they cause such as encephalitis, febrile illness accompanied by rash and arthritis as well as haemorrhagic fever. Infections can cause a wide range of mild or severe symptoms with significant morbidity and mortality especially in tropical countries (Becker et al., 2003).

Dengue and dengue haemorrhagic fever which are primarily transmitted by the mosquito *Ae. aegypti*, constitute increasingly an important burden to mankind in terms of morbidity and mortality. About 1.5 billion people in the tropics, mainly in Asia, the Western Pacific region, the Caribbean, as well as Central and South America, live under the risk of dengue virus infection (Halstead, 1980; 1982; 1992; Becker et al., 1991; Gratz, 1999).

Besides the clinical symptoms, records of arboviruses are based on virus isolation from mosquitoes and vertebrates including the sentinel-method (serologicaly negative vertebrates, *e.g.* rabbits or chickens, are exposed to mosquito bites in the field; after a while the blood is tested for virus antibodies) as well as serological investigations to detect virus antibody in vertebrates including humans. More recently molecular techniques *e.g.* polymerase chain reaction (PCR) have been applied (Puri et al., 1994).

1.3.3. Filariasis

Vertebrates are the primary hosts for all filarial nematodes. They are the hosts for adult worms, and sexual reproduction occurs within these hosts. The tissues in which adult worms occur characterize the disease caused by the worms. Adult *Wuchereria bancrofti* remain in the lymphatic vessels and lymph nodes, and the disease caused by these and similar parasites is called lymphatic filarisis (Eldridge et al., 2000).

In the tropics, lymphatic filarial diseases caused by the nematodes *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* affect an estimated 120 million people in Asia, Africa and South America; 90% of the infections are caused by *W. bancrofti*. An estimated 905 million people are directly exposed to the infection by transmission of various genera of mosquitoes, the most important are *Cx. quinquefasciatus* and *Mansonia* spp. (Becker et al., 2003).

Mosquitoes can become infected by feeding on the peripheral blood of microfilaremic hosts. In all cases, larval development takes place in the indirect flight muscles. As with all filariae, the duration of vector phase development in the lymphatic dwelling parasites is a function of ambient temperature and usually ranges from 8-16 days. Third stage larvae (L3) are transferred to the host from the mouthparts of the mosquito during a subsequent bloodfeeding (Eldridge et al., 2000).

1.4. Significance of the study

The general objective of the Ph. D. work is to increase the knowledge on the bionomics of nuisance and vector mosquitoes. Specifically, it aims: 1) to identify by protein electrophoresis the genetic variation between sibling species or subspecies as vector or nuisance mosquitoes; 2) to investigate the ultrastructure of mosquito ovaries as a basis for the identification of

Wolbachia sp. infections; 3) to investigate the presence of *Wolbachia* sp. infections in mosquito populations from Germany and the Philippines in order to have a better understanding on the reproduction of mosquitoes infected with *Wolbachia* sp.; 4) to assess the prevalence of dengue viruses among Filipino patients in order to emphasize the biomedical role of *Aedes aegypti* as vector of dengue as well as to support the implementation of vector control programmes and 5) to support the implementation of new biological control tool in order to combat mosquito borne diseases.

2. Protein electrophoretic study of mosquitoes (Diptera: Culicidae)

2.1. INTRODUCTION

2.1.1. Molecular and biochemical methods in taxonomy

Some mosquito groups can hardly be differentiated and are more oftenly indistinguishable by morphological traits especially members of species complexes as the Culex Pipiens Complex. In order to obtain more information on their taxonomic differentiation, biochemical and molecular methods are applied since the 1960‘s. Both DNA and gene products are used for this purpose. Gene products (proteins) are heritable traits and differences in protein structure reflect the genetic differentiation on DNA-level due to the colinearity of encoding gene sequences and the resulting amino acid sequences of the gene products.

Proteins are charged particles with different sizes and shapes. For this reason they can be distinguished in an electrical field by gel electrophoresis and mainly blood proteins and enzymes are being used. Enzymes are excellent diagnostic characters, particularly valuable for the identification of individuals or morphologically nearly indistinguishable species. Many enzymes are genetically polymorphic hence, individuals of the same population may differ from each other in their enzyme pattern. The differences between populations by considering the variation in allele frequencies are taxonomically significant.

Elucidation of the genetics of enzyme variability in culicine mosquitoes continues to contribute immensely to the understanding of the molecular biology and the vector competence of various species and populations (Laven, 1967; Wagner & Selander, 1970; Tibayrene, 1979; Gubler et al., 1982). The detectable biochemical differences between species make electrophoretic techniques of great value in describing and identifying members of different species. The application of electrophoretic techniques to identify variable (polymorphic) gene loci in mosquitoes is a suitable tool in mosquito taxonomy. Electrophoretic markers in mosquitoes are useful for formal genetics (genetic maps), release experiments and the study of reproductive behaviour (multiple insemination, mating propensity, effective population size, etc.). Such investigations are considered especially useful in view of the present attempt of genetic control (Bullini & Coluzzi, 1972a). These

techniques have been available since 1961 and are now widely used in the study of the genetics and evolutionary biology of a wide range of organisms. Electromorphic and genetic variability within several enzyme systems has been reported in the Culex Pipiens Complex by several authors (Simon, 1969; Garnett & French, 1971; Narang et al., 1977; Pasteur et al., 1981; Pryor et al., 1979; Pryor & Ferrell, 1981; Igbokwe, 1988; Igbokwe & Braden, 1989).

Electrophoretic techniques are able to distinguish multiple fractions of proteins migrating through a gel matrix under the influence of an electric current. The electrophoretic study of gene-enzyme systems represents a powerful tool in research fields of genetics, evolutionary biology, systematics, ecology and applied zoology. Various recent studies on gene-enzyme systems deal with diptera, particularly the genus *Drosophila.* Due to their ecological and medical importance, a number of contributions are also available for mosquitoes.

There are actually two forms of protein data that can be obtained simultaneously by using electrophoretic methods. One is derived from isozymes, which are functionally similar forms of enzymes, including all polymeres of sub-units produced by different gene loci (mostly paralogous traits) or by different alleles at the same locus (homologous traits) (Markert & Moller, 1959). The latter data consists of allozymes, a subset of isozymes, which are variants of polypeptides representing different allelic alternatives of a distinct gene locus (Murphy et al., 1995).

Proteins have been studied by a number of techniques: SDS electrophoresis, two dimensional chromatography and electrophoresis, immunology, "finger-printing" of peptides and amino acid sequencing. However, most of the work has been done by using the standard methods of gel electrophoresis because this technique can be easily performed and is among the most cost-effective methods of investigating genetic phenomena at the molecular level (Ferguson, 1980).

Protein electrophoresis is defined as the migration of proteins under the influence of an electrical field (Pasteur et al., 1981). Proteins are composed of amino acids joined by covalent peptide bonds to form polypeptides. These sequences, or primary structures, are genetically determined. Each of the 20 eukaryotic amino acids has a unique side chain, characterized by its shape, size, and charge. The side chains on five of these amino acids are either basic (potentially positively charged), or acidic (negatively charged) depending on their

environment. The amino acid sequences of proteins may be altered by mutations in the encoding DNA locus. Resulting amino acid exchanges may alter the shape and net charge, as well as the catalytic efficiency and stability of an enzyme.

Polypeptides migrate through an electric field at different rates, according to their net charge, size and shape. Proteins which migrate different distances usually differ by at least one amino acid. The colinearity of amino acid sequence and nucleotide sequence in the DNA imply that these proteins were encoded by segments of DNA differing in at least one base pair. Thus, electrophoretic mobility of proteins provides indirect information on the DNA.

2.1.2. Morphology and ecology of the Culex Pipiens Complex

Moquitoes of the Culex Pipiens Complex include a number of closely related taxa, showing extensive variation in their morphological and ecological characteristics (Urbanelli et al.,1995). The Culex Pipiens Complex includes the following subspecies, namely; *Culex pipiens quinquefasciatus* Say*, Cx. p. pipiens* Linnaeus, *Cx. p. pipiens* biotype *molestus* Forskal*, Cx. p. phytophaga* Ficalbi*, Cx. p. comitatus* Dryar and Knab*, Cx. p. dipseticus* Dryar and Knab*, Cx. p. pallen* Coquillett*, Cx. p. australicus* Dobrotworsky and Drummond*, Cx. p. globocoxitus* Dobrotworsky*, Cx. p. berbericus* Roubaud *and Cx. p. torridus* Iglisch.

Culex p. pipiens and *Cx. p. quinquefasciatus* are the most widespread members of the Culex Pipiens Complex, the former being present in temperate regions and the latter in tropical regions. Zones of overlap and/or intergradation in the subtropics have been extensively studied in North America and Japan and have also been reported in areas of the Middle and Far East , South America, Australia and Africa (Urbanelli & Bullini, 1985).

There are different features or characteristics that have been used to distinguish between the typical *Cx. p. pipiens* and *Cx. p. quinquefasciatus* including such morphological features as colour, scale patterns, wing venation, aedeagus morphology, shape of the pupal trumpets, larval siphon indices, branching of the siphonal tufts and the lateral and transutural setae, as well as the biological characteristics of mating behaviour, host preferences, occurrence of adult diapause and larval habitat (Barr, 1982). However, as Belkin (1962) states, that the only reliable diagnostic character up to the present has been the phallosome morphology of the male genitalia. The differences of this character involve (a) the shape of the tips of the pair of dorsal arms of the phallosome, which are thin and pointed in *Cx. p. quinquefasciatus* and

thick and blunt in *Cx. p. pipiens*; (b) the allignment of the dorsal arms which are more or less parallel in *Cx. p. quinquefasciatus* and divergent in *Cx. p. pipiens*; and (c) the shape of the ventral arms of the phallosome which are widely expanded in *Cx. p. quinquefasciatus* and narrow in *Cx. p. pipiens*. The latter two features are generally combined in a single measure, expressed as the DV/D ratio, where DV is the distance between the tips of the dorsal and ventral arm of the male genitalia, and D is the distance between the tips of the dorsal arms (Sundararaman, 1949). This ratio has been proven to be reliable by several authors (Barr, 1957; Bullini & Coluzzi, 1973; Tabachnick & Powell, 1983; Humeres et al., 1990).

The name *Culex p. pipiens* dates from the 10th edition of Linnaeus' *Systema Naturae* in 1758 in which the distribution was given in Europe and America. This polytypic species of the subgenus *Culex* is one of the most widely distributed of all mosquitoes. It occurs around the world between latitudes of 60°C North and 40° South and it has been extensively studied because of its parasitological interest and its complex taxonomic status (Bullini & Coluzzi, 1978). Among the populations of *Cx. p. pipiens*, the biological form known as '*autogenicus* Roubaud' or '*molestus* Forskal' is characterized by autogeny, stenogamy and other features, but these forms have been synonymized with *Cx. p. pipiens* (Barr, 1981).

Culex p. quinquefasciatus was described by Thomas Say in 1823 from western United States which at that time extended only to the Mississippi River. The type of locality was later restricted to the vicinity of New Orleans (Belkin et al., 1966) .

2.1.3. Genetics in taxonomy of mosquitoes

Populations belonging to different species almost invariably oftenly show considerably more genetic differences than con-specific populations. Morphological characters are mainly formed by selection pressure while protein characteristics are more plastic and their variability is influenced by chance besides selection. Consequently, divergence of protein traits is possible even when no morphological differentiation between taxa is detectable. In this case not only polymorphic gene loci with shared alleles, but also monomorphic loci with fixed alleles in each population, contribute to observed genetic distances, since species are frequently monomorphic for different alleles. Biochemical differences between species make electrophoretic studies of great value in describing and identifying members of the closely related taxa of the Culex Pipiens Complex.

The *Culex peus* population is not a member of the Culex Pipiens Complex. All developmental stages are well differentiated from the *Culex pipiens* species by morphological characters. It is included in the study because of it's clear taxonomic status and it serves as an outgroup taxon for the evaluation of the genetic differentiation between the *Culex pipiens* populations.

The study aims to identify the two sibling species or subspecies, namely: *Cx. p. pipiens* and *Cx. p. quinquefasciatus* by using electrophoretic analysis of gene-enzyme systems in order to emphasize the medical as well as the epidemiological importance of these mosquitoes complex as vectors of several parasites.

2.2. MATERIALS AND METHODS

2.2.1. Mosquito strains

A total of 668 mosquito individuals were used in the study. The individuals were composed of both sexes because no differences in allele frequencies were observed between males and females (Urbanelli et al., 1995). These were composed of *Culex. p. pipiens, Culex p. quinquefasciatus* and *Culex peus.* The mosquitoes were sampled during larval stage from three different countries. Populations of *Cx. p. quinquefasciatus* were collected from Cebu City, Philippines as well as from Los Angeles, USA. The population of *Cx. peus* was sampled from the same breeding site as *Cx. p. quinquefasciatus* in Los Angeles, USA and the population of *Cx. p. pipiens* were collected from Lahr, Germany.

2.2.2. Collection of mosquito larvae

Larvae were sampled in the field and reared until adult stage in the laboratory. The collection was done by scooping the mosquito larvae in the water with a fine mesh net. Larvae and adult mosquitoes (48 h after emergence) were stored in liquid nitrogen until use for electrophoresis.

2.2.3. Mode of rearing the mosquito larvae under laboratory conditions

All larvae were raised and held in the laboratory at a temperature of 26 ±°C and relative humidity between 70% and 80%. The larvae were reared in a round enameled pans (30 cm diameter by 9 cm deep) with corresponding labels according to their localities. Each pan was filled up with 2,000 mL of pond water and the larvae were allowed to developed until pupal

stage. The principal food of these mosquito larvae was the detritus or particulate matters that were present in the water. A small amount of pond water (about 100 mL) was added daily in each enameled pan in order to sustain the principal food. Liver powder was also added in small amounts to avoid malnutrition of the larvae. The pans were covered with fine nets in order to avoid egg-laying of adult mosquitoes from different sources.

Cultures of mosquito larvae were monitored daily. Important was to avoid the growth of a bacterioneuston layer, a thin organic surface microlayer that covers the water surface and has a negative impact on oxygen intake leading to high mortality rate of the larvae (Norkrans, 1980). Therefore, the microlayers were regularly removed by scraping a tissue paper across the water surface.

Pupae were removed daily by using a small pipette and transferred into small cups with 20 mL of pond water. The cups were transferred to their corresponding cages and the adults were allowed to emerge.

Slices of apples or sucrose cubes were placed at the top of the cages in order to provide food for the adults. Adult mosquitoes, approximately two days old, were collected from the cages with a suction tube and were transferred to a 1.5 mL reaction tubes or Eppendorf vials in batches of 25 and stored in dry ice or liquid nitrogen until use for electrophoresis analysis. Some adult mosquitoes were retained in the cages in order to continue the cycle of rearing process to ensure sufficient samples for the study.

2.2.4. Enzymes

Eight enzymes were used for the protein electrophoresis in populations of *Culex* (Table 2-1).

Table 2-1. List of enzymes used in the electrophoretic study.

Enzyme	Abbreviation	E.C. No.
Adenylate Kinase	AK	2.7.4.3
Glucose-6-phosphate-dehydrogenase	GPD	1.1.1.8
Hexokinase	HK	2.7.1.1
Isocitrate-dehydrogenase	IDH	1.1.1.42
Malate-dehydrogenase (NAD-dependent)	MDH_{NAD}	1.1.1.37
Malate-dehydrogenase (NADP-dependent)	MDH_{NADP} (ME)	1.1.1.40

Mannose-phosphate-isomerase	MPI	5.3.1.8
Phosphoglucomutase	PGM	5.4.2.2

2.2.5. Preparation of samples

One mL buffer solution of 10 mM Tris/citric acid, pH 7.5 including 10 mM 2-mercaptoethanol solution was prepared. Fifteen up to 22 plastic reaction tubes (1.5 mL) were prepared with corresponding labels. Each of the vial was filled with 40 µl of buffer solution. The mosquito samples were removed from the liquid nitrogen and each one specimen was transferred into each of the 22 vials. The samples in the vials were homogenized for 15 sec. by using an ultrasound sonicator instrument (Sonopuls). The homogenate was centrifuged for a period of 1 min. at 14,000 rpm in a table Eppendorf centrifuge and the supernatants including the soluble proteins were used for electrophoresis.

2.2.6. Performance of electrophoresis

Five hundred mL of electrophoresis buffer were prepared based on the standard procedures from Harris and Hopkinson (1976) and Shaw and Prasad (1970). Table 2-2 shows the different buffer systems adapted to the enzyme specific requirements used in the the study.

Table 2-2. Buffer systems that were used for enzyme electrophoresis and gel preparation following Harris and Hopkinson (1976) and Shaw and Prasad (1970).

No.	Electrode buffer (EB)	Gel buffer	Enzyme system
1	0.25M Tris/0.057M Citric acid, pH 7.5	EB 1:4.3	MDH
2	0.25M Tris/0.052M Citric acid, pH 8.5	EB 1:4.3	ME
3	0.05M Tris/0.05M $NaH_2 PO_4$, pH 8.3	EB 1:10	MPI
4	0.1M Tris/0.1M Malic acid/10mM $MgCl_2$/10mM EDTA, pH 7.4	EB 1:10	PGM, GPI
5	0.155M Tris/0.05M Citric acid, pH 7.0	EB 1:10	AK, HK
6	0.155M Tris/0.045M Citric acid, pH 8.0	EB 1:10	IDH

2.2.7. Gel preparation

Gels were prepared using the appropriate gel buffer for the protein systems in a ratio of 1:10 (buffer : agarose + H_2O). Nine mL of the prepared electrode buffer (Table 2-2) and 0.9 g agarose (Applichem, LEEO) were filled up to 90 mL with distilled water in an Erlenmeyer

flask. The mixture was heated on a magnetic heating stirrir with continuous swirling until a vigorously boiling, clear, viscous solution was obtained (4-5 min.).

A glass plate (19x25 cm) serving as a carrier for the gel was prepared. The glass was labelled with the name of the buffer system, the date and the names of the mosquito samples. A single line was drawn (4cm) from the edge of the glass plate in order to serve as the point of origin for sample application.

The gel solution was poured onto the glass plate. It was cooled at room temperature for 10 min. until the agarose substance solidified. It was transferred into a moist box and placed immediately in the refrigerator (4°C) for a minimum of 30 min. Before putting the homogenate samples onto the gel, a longitudinal paper strip was applied (2 min.), 2 inches from 1 end of the cooled gel in order to remove the excess amount of moisture as well as it enabled the samples to penetrate the gel. A slot guide of plexiglass with corresponding numbers from 1 to 15 or to 22 was placed at the line of origin prior to electrophoresis. Three to 7 µl of the homogenate samples were pippeted from each of the fifteen vial and placed into the sample slots of the prepared gel.

2.2.8. Equipment preparation

The gel with the samples was set on the cooling plate (+4°C) and connected to the buffer tanks by electrode wicks and thus, the gel served as a bridge between both tanks. About 250 mL of electrolyte or electrode buffer were poured into each tank for an electrophoretic run. The chamber started to operate by switching on the power supply at 200 Volts for 10 min. (pre-run) and was turned to 500 Volts for 1 h (main run).

A stain solution for a specific enzyme was prepared following Harris and Hopkinson (1976) and Murphy et al. (1996). Each enzyme used in the experiments had a corresponding stain (Table 2-3).

Table 2-3. The different enzyme systems with their corresponding stains.

Enzyme	Abbreviation	E.C. No.	Stain Composition	Quantity
Malate-dehydrogenase (NAD)	$MDH_{(NAD)}$	1.1.1.37	0.1 M Tris/HCl, pH 8.0	25 ml
			L-Malic Acid	350 mg, pH 8.0
			NAD	10 mg
			MTT	7.5 mg
			PMS	5 mg
			Agar, 2 %	25 ml
Enzyme	**Abbreviation**	**E.C. No.**	**Stain Composition**	**Quantity**
Malate-dehydrogenase (NADP)	$MDH_{(NADP)}$, (ME)	1.1.1.40	0.1 M Tris/HCl, pH 7.0	25 ml
			L-Malic acid	100 mg, pH 7.0
			$MgCl_2$, 1M	0.5 ml
			NADP	5 mg
			MTT	5 mg
			PMS	0.5 mg
			Agar, 2%	25 ml
Isocitrate-dehydrogenase	IDH	1.1.1.42	0.5 M Tris/HCl, pH 8.0	25 ml
			Isocitric acid	20 mg
			$MgCl_2$, 1M	3 ml
			NADP, MTT, PMS	5 mg each
			Agar, 2%	25 ml
Hexokinase	HK	2.7.1.1	0.05 M Tris/HCl, pH 7.1	25 ml
			$MgCl_2$*6H2O	9 mg
			NADP	5 mg
			Glucose	40 mg
			ATP	10 mg
			G6PDH	35 µl (80u)
			MTT	8 mg

			PMS	2 mg
			Agar, 2%	25 ml
Enzyme	**Abbreviation**	**E.C. No.**	**Stain Composition**	**Quantity**
Adenylate Kinase	AK	2.7.4.3	0.05 M Tris/HCl, pH 7.1	20 ml
			$MgCl_2*6H_2O$	9 mg
			NADP	5 mg
			Glucose	40 mg
			ADP	8 mg
			Hexokinase	0.08 µl (160u)
			G6PD	35 µl (80u)
			MTT	8 mg
			PMS	2 mg
			Agar, 2%	30 mL
Mannose-phosphate-isomerase	MPI	5.3.1.8	0.2 M Tris/HCl, pH 7.5	25 ml
			Mannose-6 phosphate (Ba-Salz)	10 mg
			1 M $MgCl_2$	0.2 ml
			Glucose-phosphate-isomerase	2 µl
			Glucose-6-phosphate-dehydrogenase	3 µl
			NADP, MTT, PMS	5 mg
			Agar, 2%	25 mL
Glucose-6-phosphate-isomerase	GPI	5.3.1.9	0.2 M Tris/HCl, pH 8.0	25 ml
			1 M $MgCl_2$	0.4 ml
			Fructose-6-phosphate (Ba-Salz)	20 mg
			Glucose-6-phosphate-dehydrogenase	2.5 µl
			NADP, MTT, PMS	5 mg each
			Agar, 2%	25 ml

Enzyme	Abbreviation	E.C. No.	Stain Composition	Quantity
Phosphoglucomutase	PGM	2.7.5.1	0.05 M Tris/HCl, pH 8.0	25 ml
			Glucose-1-phosphate	40 mg
			Glucose-1,6-diphosphate	0.1 mg
			$MgCl_2$, 1 M	0.5 ml
			Glucose-6-phosphate-dehydrogenase	2.5 µl
			NADP, MTT, PMS	5 mg
			Agar, 2%	25 ml

The individual proteins had to be selectively stained and thus, a specific substrate for the enzyme was desired to allow the catalyzation of the particular reaction. The development of a dye, visualized the formation of bands of enzyme activity in the gel when electrophoresis was successful. The stain solution including substrate, co-factors and dye was poured into the gel and was immediately incubated in the oven (37°C). The results were observed (the earliest was 20 min.) by the appearance of the different bands caused by enzyme activity in the gel. Photoes were taken from these results and the migration of different isozymes were also recorded and drawn for the interpretation of data.

2.2.9. Data nomenclature and analyses

The appearance of enzyme bands in the gels with different electrophoretic mobilities from different samples implies that these electrophoretic variants of enzymes are encoded either by different alleles (allozymes) or by different gene loci (isozymes), respectively. The appearance of one band or single band implies a gene locus whose gene products are electrophoretically indistinguishable and that no allelic variation is detectable. These gene loci are considered as monomorphic and homozygous. In contrary, the appearance of more than one band implies heterozygosity depending on the quarternary structure of the protein, or homozygote at more than one locus. After the representation of the alleles, a hyphenated number (if the enzyme is encoded by more than one locus) is assigned according to their electrophoretic mobility (no. 1 is closer to the origin, i.e. less mobile, than no. 2). Allelic variants of enzymes are assigned by a subscript number depending on their relative electrophoretic mobility while the mobility of the most common allozyme of the reference population (*Cx. p. pipiens*, Germany) is expanded to the value of 100 mm. The relative

mobility of each other allozyme is expanded by the same factor (e.g. IDH-2*70, IDH-2*100 and IDH-2*120, where IDH-2*100 is the most common allozyme with intermediate electrophoretic mobility).

The zymograms or the allozyme patterns on gels obtained after electrophoresis depend on the structure of the enzymes (Evans, 1987). A monomeric enzyme consists of one polypeptide chain, thus, an electrophoretic detectable heterozygous individual possesses two bands. First band corresponds to the position of one homozygote and the second band corresponds to the other homozygote. For enzymes which consist of two polypeptides (dimeric structure such as IDH), heterozygotes are represented by three bands with the middle band usually being the strongest due to the combination of both variants in one protein molecule. Nevertheless, monomorphic enzymes or homozygous gene loci are always represented by one band except of post-translational (non-genetic) modifications.

Furthermore, the band patterns that appeared in the gels represent the phenotypes with a clear genetic background. Allozymic variation reflects a part of the genetic variation on DNA-level. Diploid organisms such as mosquitoes consist of two copies of each gene locus (except of the few genes of the sex-chromosomes in males). The products of both gene copies can be identical (monomorphic) or can differ in amino acid sequence and the underlying variants of the same gene locus are called alleles. The genotype describes the combination of identical or different alleles in one specimen. Genotypic frequencies in a certain population depend on the allele frequencies of that population.

The degree of heterozygosity reflects the combination frequencies of different alleles and provides a measure of the genetic variability within the population. This is calculated by adding up the numbers of each heterozygous genotype at a given locus and dividing each by the total number of all genotypes combined (total sample).

Beside the genotypic frequencies, the frequencies of each allele at each locus were calculated. This was done by counting the number of times each variant appeared and dividing it by the total number of distinguishable alleles in the sample. The frequency of an allele calculated in this way is the frequency of individual homozygous for that allele, plus half the frequency of heterozygotes for that allele. Each allele frequency was calculated separately and when totalled the sum should be equal to 1.0. This was done by using the formula below (Evans, 1987).

$$pA = \frac{N_{AA} + 1/2N_{AB}}{N_{total}} \quad ; \quad pB = \frac{1/2N_{AB} + N_{BB}}{N_{total}}$$

where *A*, *B* are allele frequencies; *N* is the sample size.

Allele frequencies were obtaind from the randomly choosen samples out of the whole population. They accurately reflect the frequencies in the population as a whole, particularly if the samples are large enough in number. In this case, it is of great importance to calculate the variance of each allele frequency as a measure of accuracy for the estimated real population's status. Formula for doing this together with an algebraic representation of the above method for estimating allele frequencies is given below (Evans, 1987).

$$\text{Var } (pA) = pA\ (1\text{-}pA)/2\ N_{\text{total}}$$

where Var (*pA*) is the variance of allele frequency p*A* of allele *A* and N_{total} is the size of the mosquito sample.

The calculation of the Confidence Interval was taken from Weir (1990) for the probability of 95%.

$$\text{CI}(pA) = pA \pm 2\sqrt{\text{Var } (pA)}$$

By 5% probability, a randomly choosen sample posseses allele frequencies beyond the calculated interval.

2.2.10. The Hardy-Weinberg Equilibrium

The Hardy-Weinberg Equilibrium was founded by Hardy and Weinberg in the year 1908. It states that in a randomly mating population, the expected distribution of genotypes is determined by the random combination of alleles and this results in an equilibrium being set up among the genotypic frequencies at any given locus that remains constant from one generation to the other.

For the proof of the individual genotypic frequencies, the expected frequency values of each genotype have to be determined. The Hardy-Weinberg formula states for homozygote genotypes Npi^2 and *2Npipj* for the heterozygote genotypes. In case of a limited number of individuals (N<100 individuals, accdg. to Spiess, 1989) the number of homozygotes is over

represented and has to be corrected (Levene, 1949; Haldene, 1954; Smith, 1970). The genotype frequencies are corrected according to the following formula (Lessios, 1992):

$$E_{ij} = \frac{4N^2 p_i p_j}{2N-1} \quad \text{for heterozygote}$$

$$E_{ii} = \frac{Np_i(2Np_i - 1)}{2N-1} \quad \text{for homozygote}$$

In order to prove whether the observed genotype distribution is corresponding to the expected genotype distribution of a Hardy-Weinberg population or a significant difference is given, a χ^2 test was done according to the following formula (Lessios, 1992):

$$\chi^2 = \sum_{i<j}^{n} \frac{\left(\left|Oi_j - Ei_j\right| - c\right)^2}{Ei_j} + \sum_{i=1}^{n} \frac{\left(\left|O_{ii} - E_{ii}\right| - c\right)^2}{E_{ii}}$$

c refers to the correction factor which amounts to 0.25 if two alleles or is zero if three and more alleles are considered (Emigh, 1980).

As for the estimated allele frequency the degree of freedom is reduced to one. The degree of freedom is calculated in this test not according to the genotypes –1, but acording to the following formula (Dobzhansky & Levene, 1948; Crisp, 1978).

$$FG = \frac{n(n-1)}{2}$$

n = number of alleles at the given locus.

In order to have a valid data, it was assumed according to Cochran (1954) that the expected frequency is greater than 1 and at the same time 60% of the frequencies are higher than 5. In the χ^2 test, all rare alleles were combined and the new frequencies were computed. In case one locus had 2 alleles icluding one rare allele, a table after Vithayasai (1973) was used to determine the heterozygosity in relation to the sample size and the frequency of the rare alleles.

2.2.11. Genetic identity and distances

The genetic distances between two populations for one gene locus can be determined according to Nei (1972) after the following formula:

$$I_L = \frac{\sum x_i y_i}{\sqrt{\sum x_i^2 \sum y_i^2}}$$

For the average of all determined loci, the following formula has been applied:

$$I_{\varnothing} = \frac{J_{xy}}{\sqrt{J_x J_y}}, \qquad \text{where } J_{xy} = \Sigma\Sigma x_i y_i,\ J_x = \Sigma\Sigma x^2_i,\ J_y = \Sigma\Sigma y^2_i$$

The genetic identity is expressed in numbers between zero (populations don't have any common allele) and one (the populations have identical allele frequencies).

The genetic distance D is calculated by

$$D_{\varnothing} = -\ln I_{\varnothing}$$

according to Nei (1972). *D* varies between zero for populations having the same frequencies until indefinite (theoretically) for populations with no common alleles. The genetic distances between the *Culex* populations were calculated using Gendist from the Phylip programme package (Phylgeny inference package, windows version 3.6C) by Felsenstein (2002).

The UPGMA method

For a better documentation of the calculated paired distances between the populations, dendrograms were constructed based on the UPGMA-cluster analyses (Unweighted Pair Group Method with Arithmetic Means) with the Neighbor program (Phylip programme package, Felsenstein, 2002).

2.3. RESULTS

2.3.1. Results of enzyme electrophoresis in *Culex* populations

The data presented below provide indications of protein electrophoretic variability in the populations of *Culex.* The genetic variability within as well as the differentiation among the populations of *Culex* were analyzed.

Each enzyme was investigated in several electrophoretic runs in populations of *Culex*. Specific banding patterns in the gels were observed in each of the enzyme system. Original photoes were taken from these bands for documentation processes. The electrophoretic conditions were adjusted individually so that the proteins usually migrated from the origin to the top (anode). Only few variants migrated to the cathode. Allele frequencies, genotype frequencies as well as the variances in comparison between the different populations were calculated.

Oxidoreductases

Malate-dehydrogenase$_{(NADdependent)}$(MDH_{NAD})	**E.C. 1.1.1.37**

Buffer system no. 1, Running time: 66 min., Voltage: 450 V, Ampere: 105 mA, Incubation time for staining: 30 min.

The results obtained from MDH_{NAD} in populations of *Culex* showed the presence of two MDH_{NAD}- loci. These were clearly seen by the formation of bands in the gel with different electrophoretic mobilities, thus, it imply that these enzyme variants are encoded by different gene loci (Fig. 2-1A & B).

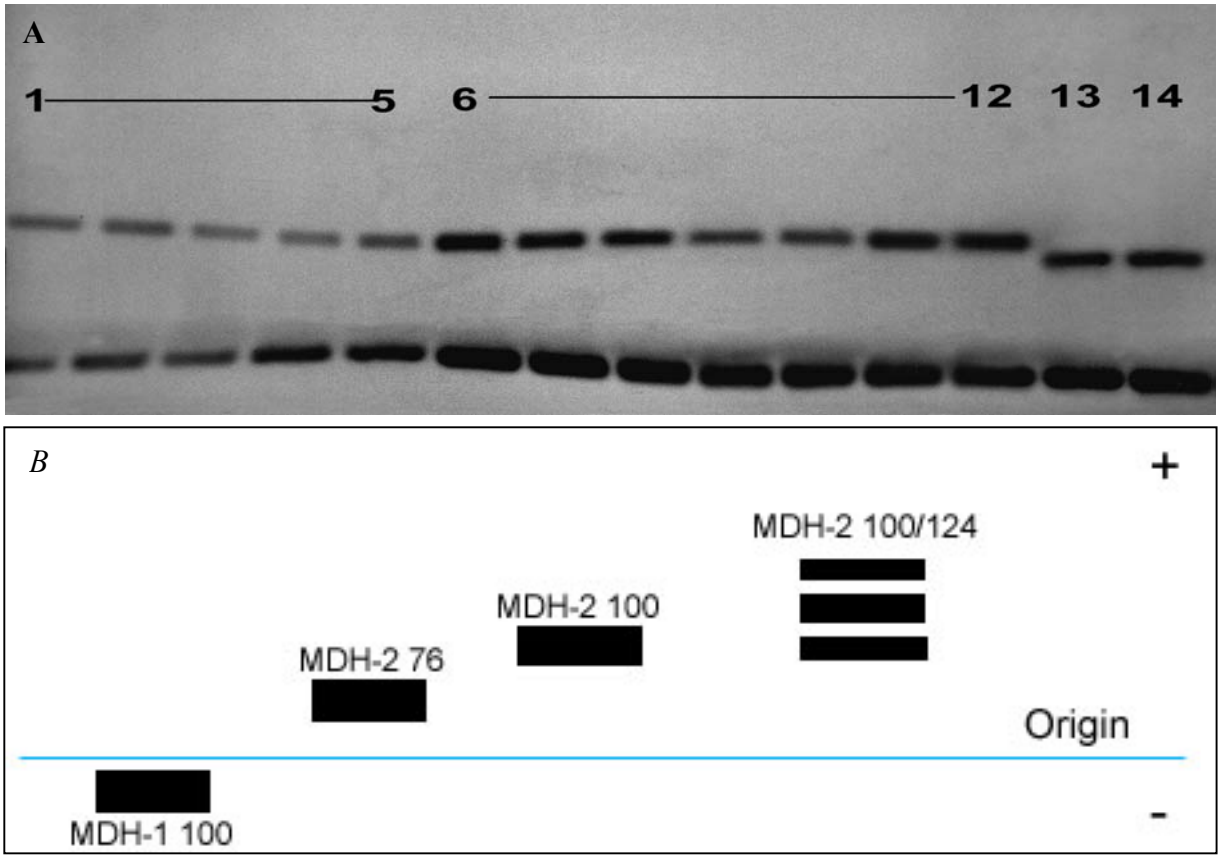


Figure 2-1A. Electrophoretic mobilities of MDH_{NAD} enzyme in populations of *Culex*. Lanes: 1 to 5: *Cx .p. pipiens*, 6 to 12: *Cx. p. quinquefasciatus* (USA), 13 to 14: *Cx. peus*. **B**. Electrophoretic mobility model of Malate-dehydrogenase (MDH_{NAD}) enzyme.

Figure 2-1A shows that only one common electrophoretic variant in populations of *Culex* was observed in MDHNAD-1* (MDHNAD-1*100). All of the investigated *Culex* populations had the same allele frequency which is equal to 1.0000.

In regards to the locus MDH-2*, three allelic variants were detected, MDHNAD-2*76, MDHNAD-2*100 and MDHNAD-2*124. *Cx. p. pipiens, Cx. p. quinquefasciatus* (Phils.) and *Culex p. quinquefasciatus* (USA), showed a common allozyme at +25 mm (MDHNAD-2*100) and a rare one at +31 mm (MDHNAD-2*124). *Culex peus* was differentiated by its own fixed allele (MDHNAD-2*76), the corresponding allozyme had a lower electrophoretic mobility (19 mm) than the *Cx. p. pipiens* reference allozyme (MDHNAD-2*100).

The absolute (Abs.) and relative (Rel.) allele frequencies as well as variances (Var.) and the confidence intervals (CI) of locus MDH-1* and MDH-2* in populations of *Culex* were calculated (Table 2-1, 2-2, 2-3 & Graph 2-1).

Table 2-1. Absolute (Abs.) and relative (Rel.) genotype frequencies of MDH_{NAD}-1* and MDH_{NAD}-2* in populations of *Culex*.

Locus	**MDH_{NAD}-1***			**MDH_{NAD}-2***						
Genotype	**100/100**		**TOTAL**	**76/76**		**100/100**		**100/124**		**TOTAL**
Population	Abs.	Rel.		Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	
Cx. p. pipiens	20	1.0000	20	0	0.0000	19	0.9500	1	0.0500	20
Cx. p. quinque-fasciatus (Phils.)	16	1.0000	16	0	0.0000	32	1.0000	0	0.0000	32
Cx. p. quinque-fasciatus (USA)	14	1.0000	14	0	0.0000	14	1.0000.	0	0.0000	14
Cx. peus	6	1.0000	6	4	1.0000	0	0.0000	0	0.0000	4

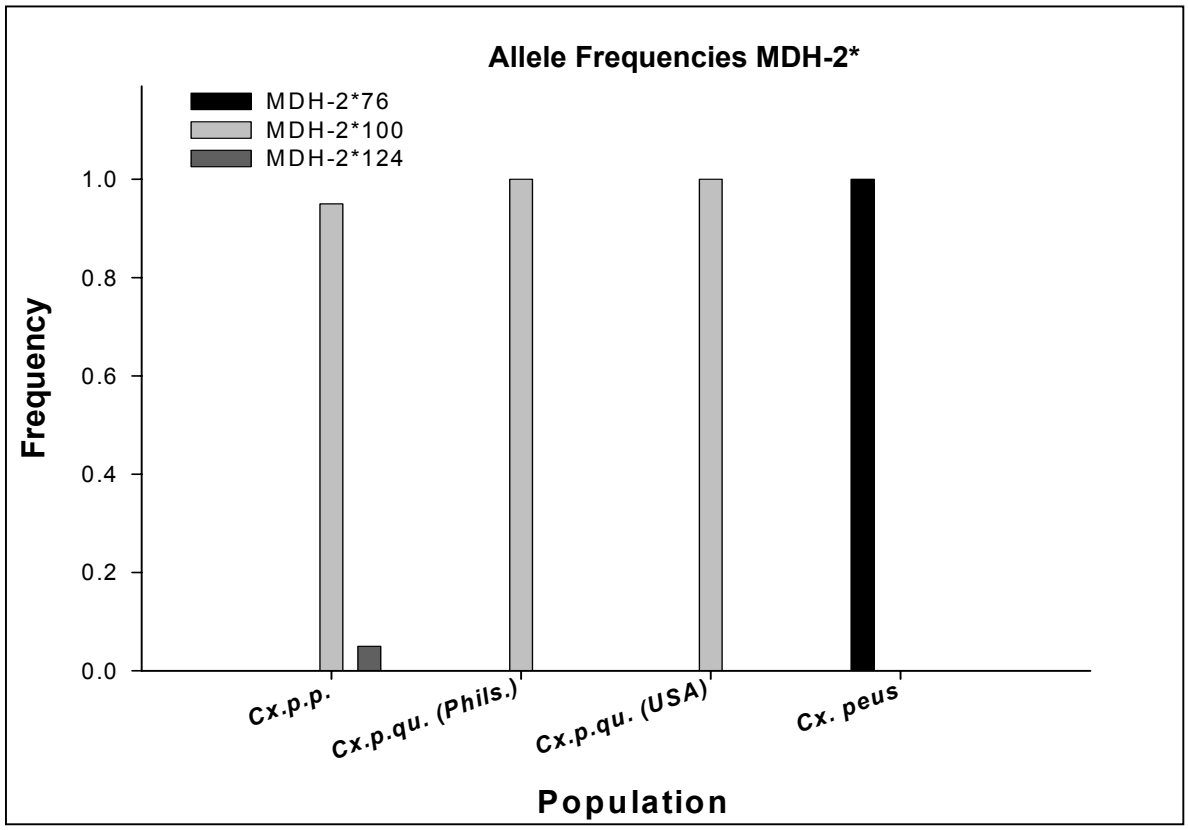
Table 2-2. Absolute (Abs.) and relative (Rel.) allele frequencies as well as variances (Var.) and confidence intervals (CI) of MDH_{NAD}-1*. The confidence interval results from the relative allele frequency ± the V-value (see 2.2.9).

Allele	**MDH_{NAD}-1* 100**			
		CI		
Population	**Abs.**	**Rel.**	**V(±)**	**Var.**
Cx. p. pipiens	40	1.0000	0.0000	0.0000
Cx. p. quinquefasciatus (Phils.)	32	1.0000	0.0000	0.0000
Cx. p. quinquefasciatus (USA)	28	1.0000	0.0000	0.0000
Cx. peus	12	1.0000	0.0000	0.0000

Table 2-3. Absolute (Abs.) and relative (Rel.) allele frequencies as well as variances (Var.) and confidence intervals (CI) of locus MDH_{NAD}-2* in populations of *Culex*. The confidence interval results from the relative allele frequency ± the V-value (see 2.2.9).

Allele	**MDH_{NAD}-2*76**				**MDH_{NAD}-2*100**				**MDH_{NAD}-2*124**			
		CI				**CI**				**CI**		
Population	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.
Cx. p. pipiens	0	0.0000	0.0000	0.0000	38	0.9500	0.0346	0.0012	2	0.0500	0.0346	0.0012
Cx. p. quinque-fasciatus (Phils.)	0	0.0000	0.0000	0.0000	64	1.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000
Cx. p. quinque-fasciatus (USA)	0	0.0000	0.0000	0.0000	28	1.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000
Cx. peus	8	1.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000

The results presented in Table 2-3 show that there were three different alleles represented by the bands MDH-2*76, MDH-2*100 and MDH-2*124. In MDH-2*124, *Culex p. pipiens* has an additional rare allele, whereas MDH-2* in *Culex p. quinquefasciatus* from the Philippines and USA was monomorphic showing a fixed common allele. On the other hand, *Culex peus* was well differentiated by one exclusive allozyme representing the allele MDH-2*76.



Graph 2-1. The distribution of allele frequencies of MDH-2* in populations of *Culex*. *Cx.. p. p.* = *Culex pipiens pipiens*, *Cx.. p. qu.*= *Culex pipiens quinquefasciatus*.

Malate-dehydrogenase$_{(NADP)}$ (MDH$_{NADP}$) (syn. Malic enzyme (ME)) **E.C. 1.1.1.40**

Buffer system no. 2, Running time: 66 min., Voltage: 450 V, Ampere: 90 mA, Incubation time: 20 min.

The dimeric enzyme of MDH$_{NADP}$ showed in all individuals that were tested an activity zone at about +40mm in anode area (Fig. 2-2 A & B).

As presented in Table 2-4, there were two MDH$_{NADP}$ allozymes obtained in all populations of *Culex*. The underlying presumptive alleles were designated as MDH$_{NADP}$*100 and MDH$_{NADP}$*107. As based on the results, *Cx. peus* showed the relative allele frequency of 0.9000 in MDH$_{NADP}$*107 while all other investigated *Culex* populations were homozygous of the allele MDH$_{NADP}$*100.

The most common allele (MDH$_{NADP}$*100) was observed both in *Cx. p. pipiens* and *Cx. p. quinquefasciatus* Philippines and USA with relative allele frequency of 1.0000 (Table 2-5 & Graph 2-2).

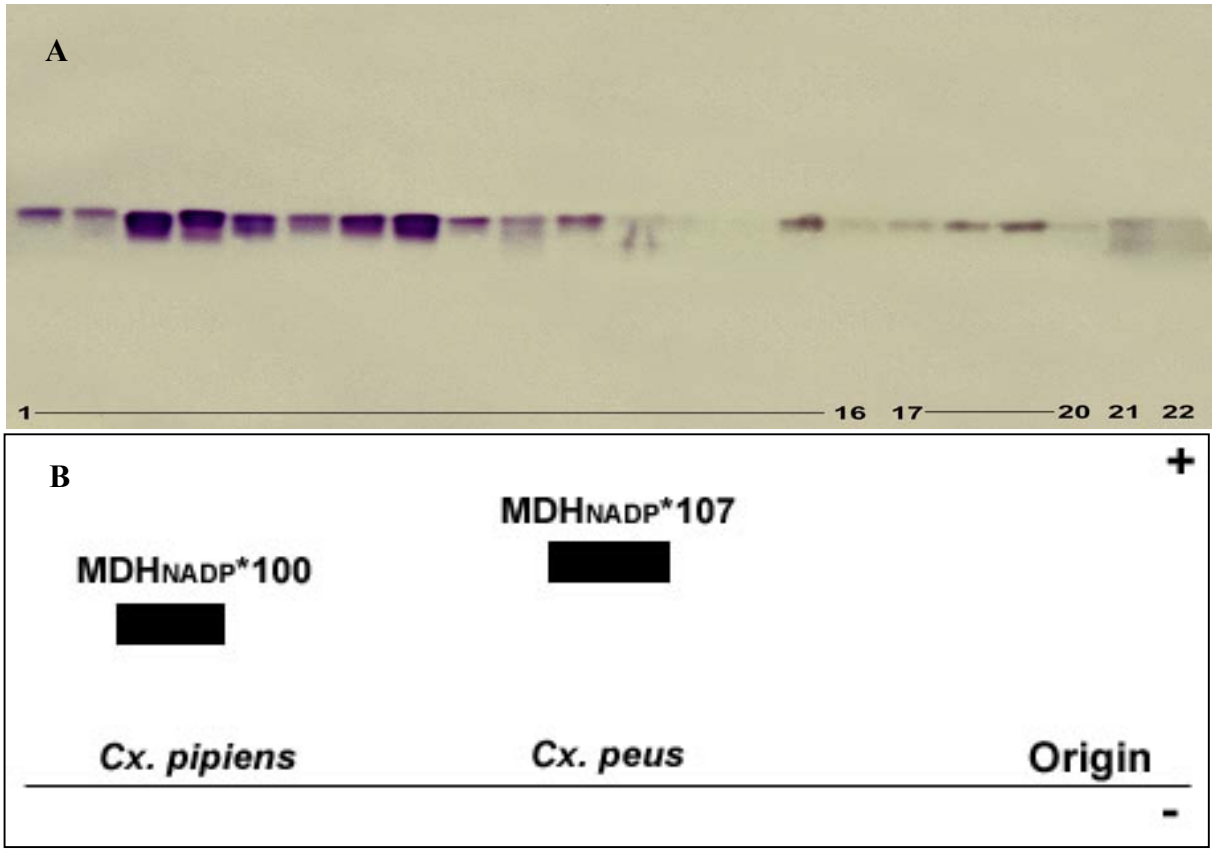


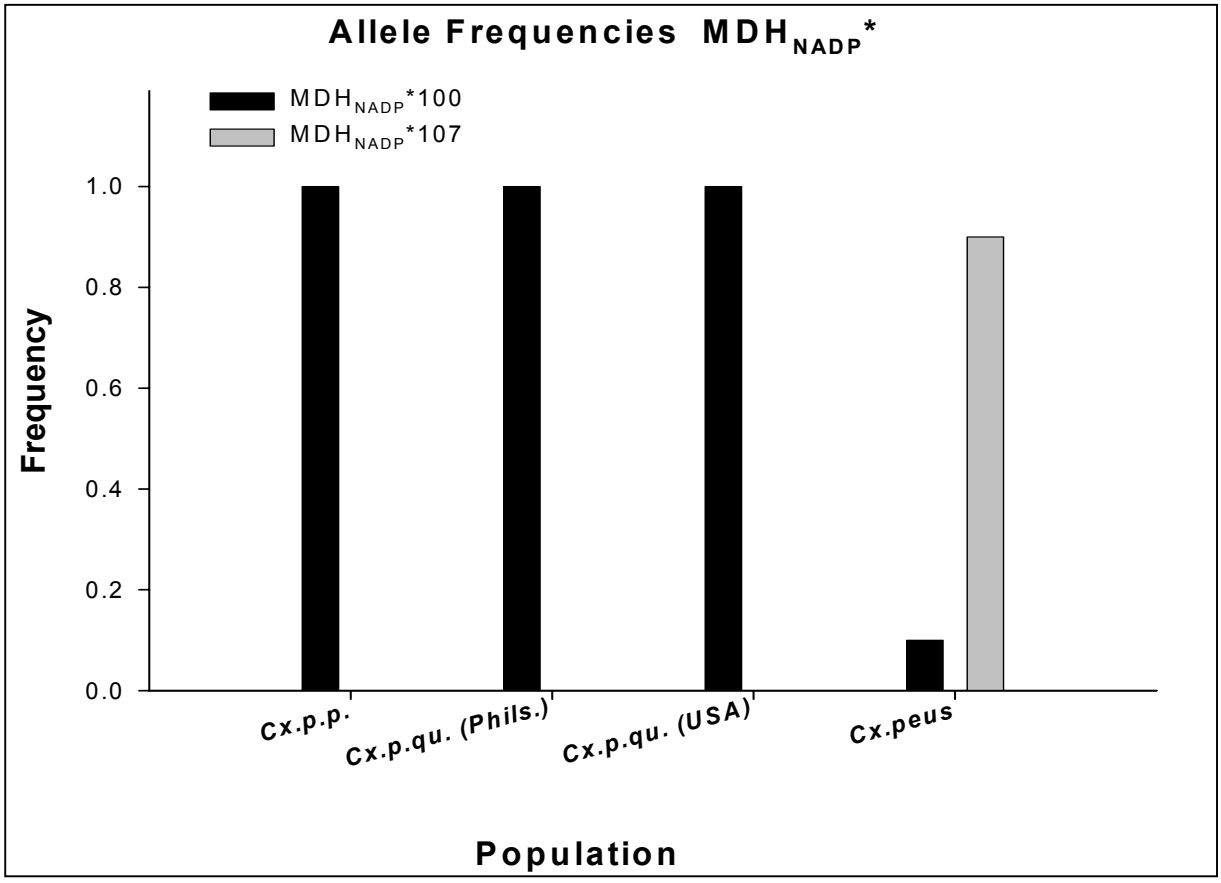
Figure 2-2 A. Electrophoretic mobilites of Malic enzyme in populations of *Culex.*1 to 16: *Cx. quinquefasciatus* (Phils.), 17 to 20: *Cx. pipiens,* 21 & 22: *Cx. peus*. **B**. Electrophoretic mobility model of Malic enzyme.

Table 2-4. Absolute (Abs.) and relative (Rel.) genotype frequencies at the gene locus MDH_{NADP}* (ME) in populations of *Culex*.

Locus	**MDH_{NADP}***						
Genotype	**100/100**		**107/100**		**107/107**		**TOTAL**
Population	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	
Cx. p. pipiens	18	1.0000	0	0.0000	0	0.0000	18
Cx. p. quinquefasciatus (Phils.)	32	1.0000	0	0.0000	0	0.0000	32
Cx. p. quinquefasciatus (USA)	14	1.0000	0	0.0000	0	0.0000	14
Cx. peus	0	0.0000	2	0.2000	8	0.8000	10

Table 2-5. Absolute (Abs.) and relative (Rel.) allele frequencies as well as variances (Var.) and confidence intervals (CI) of MDH_{NADP}* in *Culex* populations. The confidence interval results from the relative allele frequency ± the V-value (see 2.2.9).

Allele	**MDH_{NADP}*100**				**MDH_{NADP}*107**			
		CI				**CI**		
Population	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.
Cx. p. pipiens	36	1.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000
Cx. p. quinquefasciatus (Phils.)	64	1.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000
Cx. p. quinquefasciatus (USA)	28	1.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000
Cx. peus	2	0.1000	0.0671	0.0045	18	0.9000	0.0671	0.0045



Graph 2-2. The allele frequencies of Malic enzyme (MDH_{NADP}*) in populations of *Culex*.

Isocitrate-dehydrogenase (NADPdependent)(IDH) **E.C.1.1.1.42**

Buffer system no. 6, Running time: 60 min, Voltage: 500 V, Ampere: 60 mA, Incubation time: 30 min.

Isocitrate-dehydrogenase enzyme in *Culex* populations showed two codominant gene-loci, IDH-1* and IDH-2*. There were 2 alleles in IDH-1* that were observed, IDH-1*100 and IDH-1*140 (Figure 2-3 A & B). IDH-1*100 was the most frequent and the only one present in *Cx. p. pipiens, Cx. p. quinquefasciatus* (Phils.) and *Cx. p. quinquefasciatus* (USA) with a frequency of 1.0000. In contrary, this allele was not observed in *Cx. peus* samples, instead it possessed exclusively allele IDH-1*140 showing a frequency of 1.0000 (Table 2-6, 2-7 & Graph 2-3).

At the gene locus IDH-2*, three allozymes were revealed which represent at least three alleles, namely: IDH-2*72, IDH-2*84 and IDH-2*100. IDH-2*72 was present only in *Cx. p. quinquefasciatus* (Phils.). IDH-2*84 was observed in *Cx. p. pipiens, Cx. peus and Cx. p. quinquefasciatus* (Phils.) with relative frequencies of 0.0946, 0.9091 and 0.0313, respectively. However it was found that this allele was absent in the individuals of *Cx. p. quinquefasciatus*

(USA). IDH-2*100 was the commonest allele in all investigated populations of *Culex* with the frequencies of 0.9054 in *Cx. p. pipiens*, 0.8906 in *Cx. p. quinquefasciatus* (Phils.), 1.0000 in *Cx. p. quinquefasciatus* (USA) and 0.0909 in *Cx. peus* (Table 2-8, 2-9 & Graph 2-4).

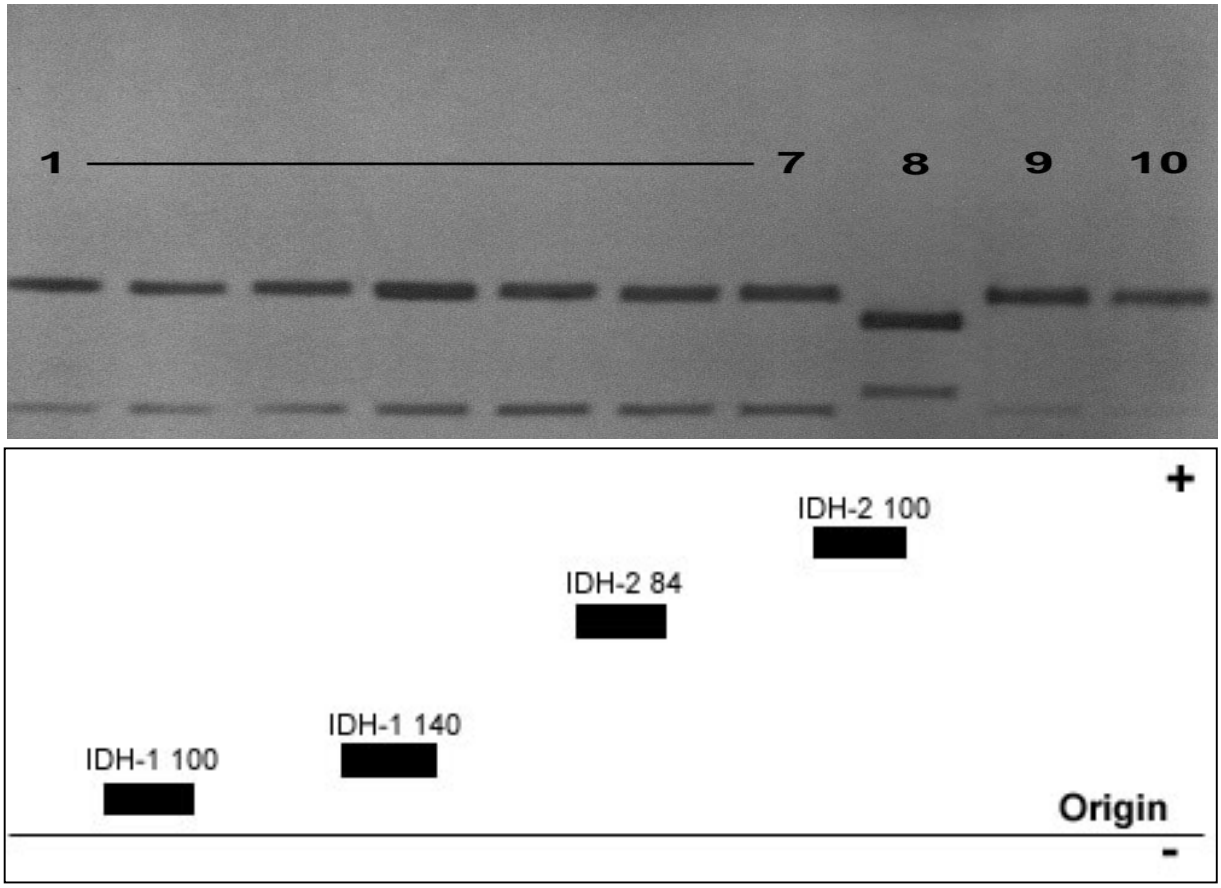


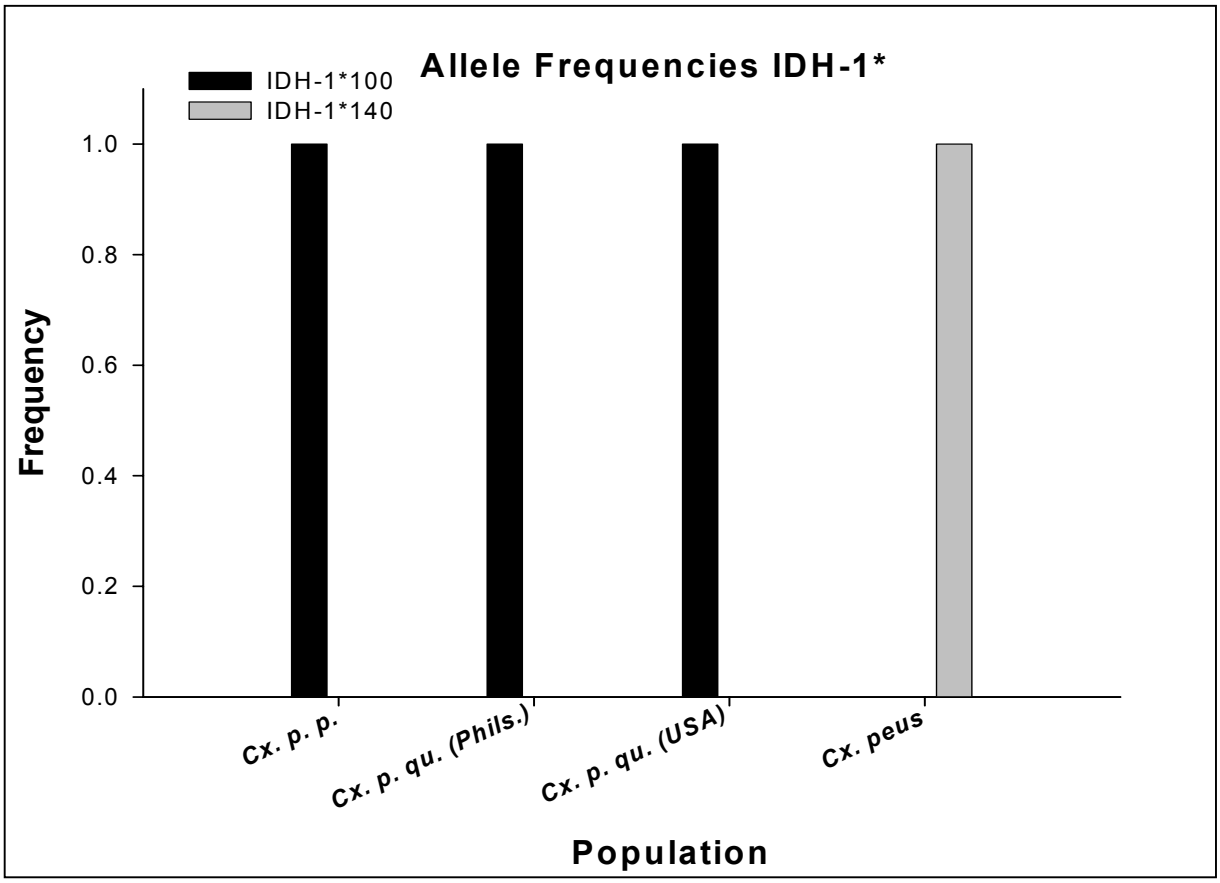
Figure 2-3A. Electrophoretic mobilities of IDH-isoenzymes in populations of *Culex*. Lanes:1 to 7: *Cx. p. quinquefasciatus* (USA), 8: *Cx. peus*, 9 & 10: *Cx. p. pipiens.* **B**. Electrophoretic mobility model of IDH.

Table 2-6. Absolute (Abs.) and relative (Rel.) genotype frequencies of IDH-1* in populations of *Culex*.

Locus	**IDH-1***				
Genotype	**100/100**		**140/140**		**TOTAL**
Population	Abs.	Rel.	Abs.	Rel.	
Cx. p. pipiens	37	1.0000	0	0.0000	37
Cx. p. quinquefasciatus (Phils.)	32	1.0000	0	0.0000	32
Cx. p. quinquefasciatus (USA)	21	1.0000	0	0.0000	21
Cx. peus	0	0.0000	11	1.0000	11

Table 2-7. Absolute (Abs.) and relative (Rel.) allele frequencies as well as variances (Var.) and confidence intervals (CI) of IDH-1* in populations of *Culex*. The confidence interval results from the relative allele frequency ± the V-value (see 2.2.9).

	IDH-1*100				IDH-1*140			
		CI				CI		
Population	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.
Cx. p. pipiens	72	1.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000
Cx. p. quinquefasciatus (Phils.)	64	1.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000
Cx. p. quinquefasciatus (USA)	42	1.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000
Cx. peus	0	0.0000	0.0000	0.0000	22	1.0000	0.0000	0.0000



Graph 2-3. Distribution of allele frequencies in IDH-1* in populations of *Culex*.

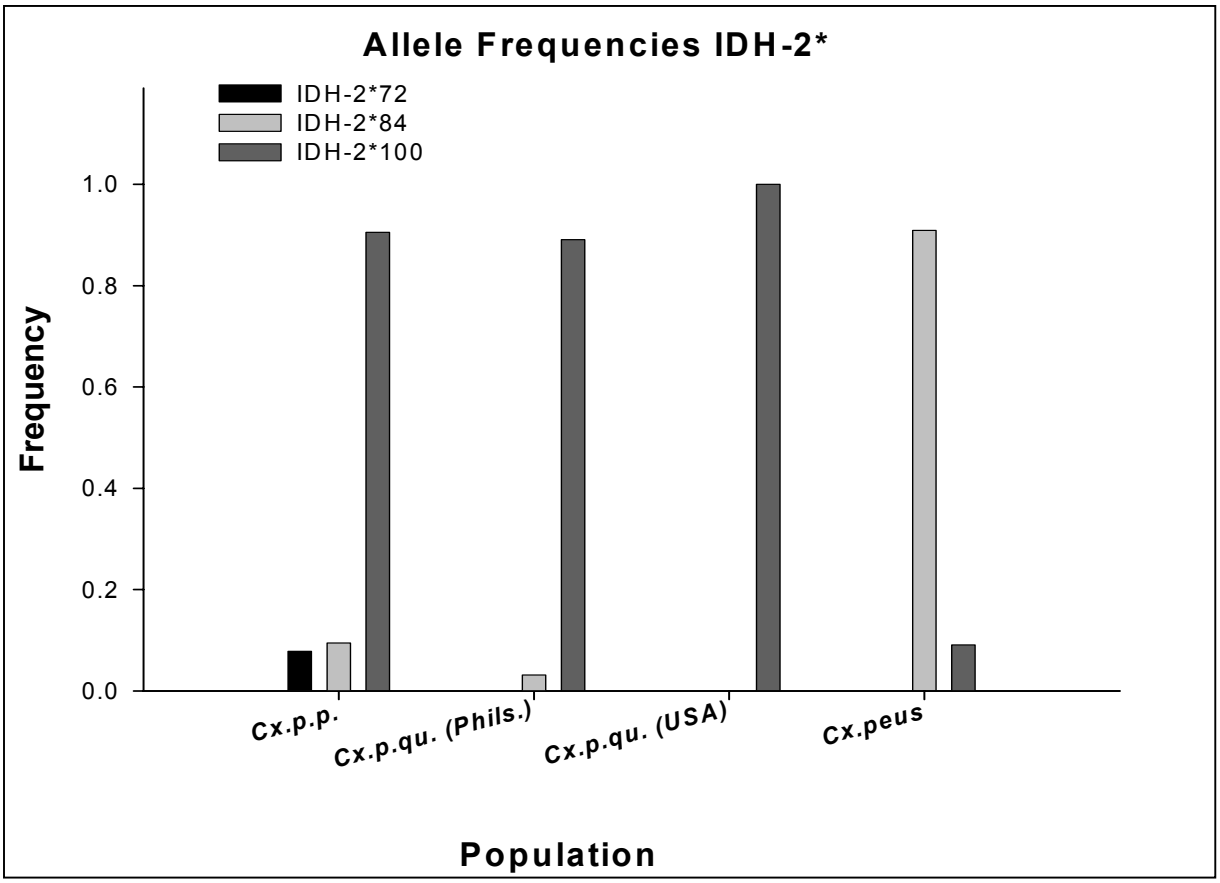
Table 2-8. Absolute (Abs.) and relative (Rel.) genotype frequencies of IDH-2* in populations of *Culex*.

Locus	**IDH-2***										
Genotype	**72/72**		**72/100**		**84/84**		**84/100**		**100/100**		**TOTAL**
Population	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	
Cx. p. pipiens	0	0.0000	0	0.0000	1	0.0270	5	0.1351	31	0.8378	37
*Cx. p. quinque-fasciatus (*Phils.)	1	0.0313	3	0.0938	1	0.0313	0	0.0000	27	0.8438	32
Cx. p. quinque-fasciatus (USA)	0	0.0000	0	0.0000	0	0.0000	0	0.0000	21	1.0000	21
Cx. peus	0	0.0000	0	0.0000	9	0.8182	2	0.1818	0	0.0000	11

Table 2-9. Absolute (Abs.) and relative (Rel.) allele frequencies as well as variances (Var.) and confidence intervals (CI) of IDH-2* in populations of *Culex*. The confidence interval results from the relative allele frequency ± the V-value (see 2.2.9).

	IDH-2*72				**IDH-2*84**				**IDH-2*100**			
		CI				**CI**				**CI**		
Population	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.
*Cx. p. p.**	0	0.0000	0.0346	0.0012	67	0.9054	0.0346	0.0012	67	0.9054	0.0346	0.0012
Cx. p. qu. (Phils.)*	5	0.0781	0.0387	0.0015	57	0.8906	0.0218	0.0005	57	0.8906	0.0387	0.0015
Cx. p. qu. (USA)*	0	0.0000	0.0000	0.0000	42	1.0000	0.0000	0.0000	42	1.0000	0.0000	0.0000
Cx. peus	0	0.0000	0.0613	0.0038	2	0.0909	0.0613	0.0038	2	0.0909	0.0613	0.0038

* *qu. = quinquefasciatus*, *p. = pipiens*



Graph 2-4. Distribution of allele frequencies in IDH-2* in populations of *Culex*.

Transferases

Hexokinase (HK)	**E.C. 2.7.1.1**

Buffer system no. 5, Running time: 60 min.,Voltage: 500 V, Ampere: 49 mA, Incubation time: 20 min.

Following the results of the HK-electrophoresis, products of three genetic HK-loci have to be assumed. Nevertheless, when variability was observed, each specimen showed the same pattern of allozymes in each banding zone, thus, inheritance of all these loci is not independent from each other (alternatively, HK isoenzymes could be the products of only one gene locus and modified after synthesis).

As based on the experiment conducted in populations of *Culex*, three isozymic forms of HK, all migrating anodally (Figure 2-4 A & B) were observed. They are referred to as the presumptive gene loci HK-1*, HK-2* and HK-3* starting from the most cathodal gene product. Each two allozymic forms have been found in *Cx. p. pipiens* for all of the three loci. In all three presumptive loci, the frequencies of genetic variants have been identical. These were alleles HK(1, 2, 3)*100 and HK(1, 2, 3)*117. Allele HK*117 was observed only in *Cx. p. pipiens* having a frequency of 0.4800. However HK*100 was found to be the commonest

allele and observed both in all investigated populations of *Culex*. This allele showed frequencies of 0.5200 in *Cx. p. pipiens* and 1.0000 in *Cx. p. quinquefasciatus* (USA), *Cx p. quinquefasciatus* (Phils.) as well as in *Cx. peus* (Table 2-10, 2-11 & Graph 2-5).

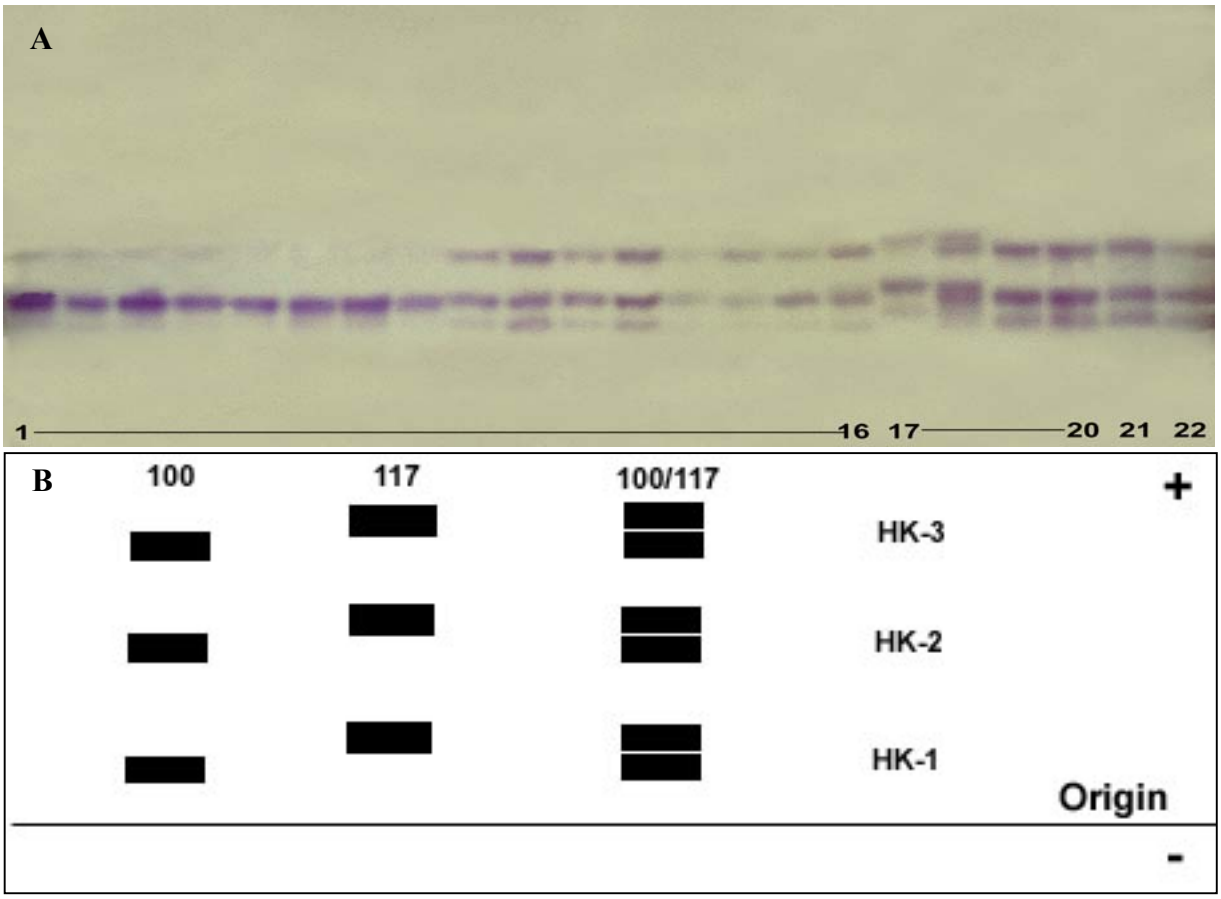


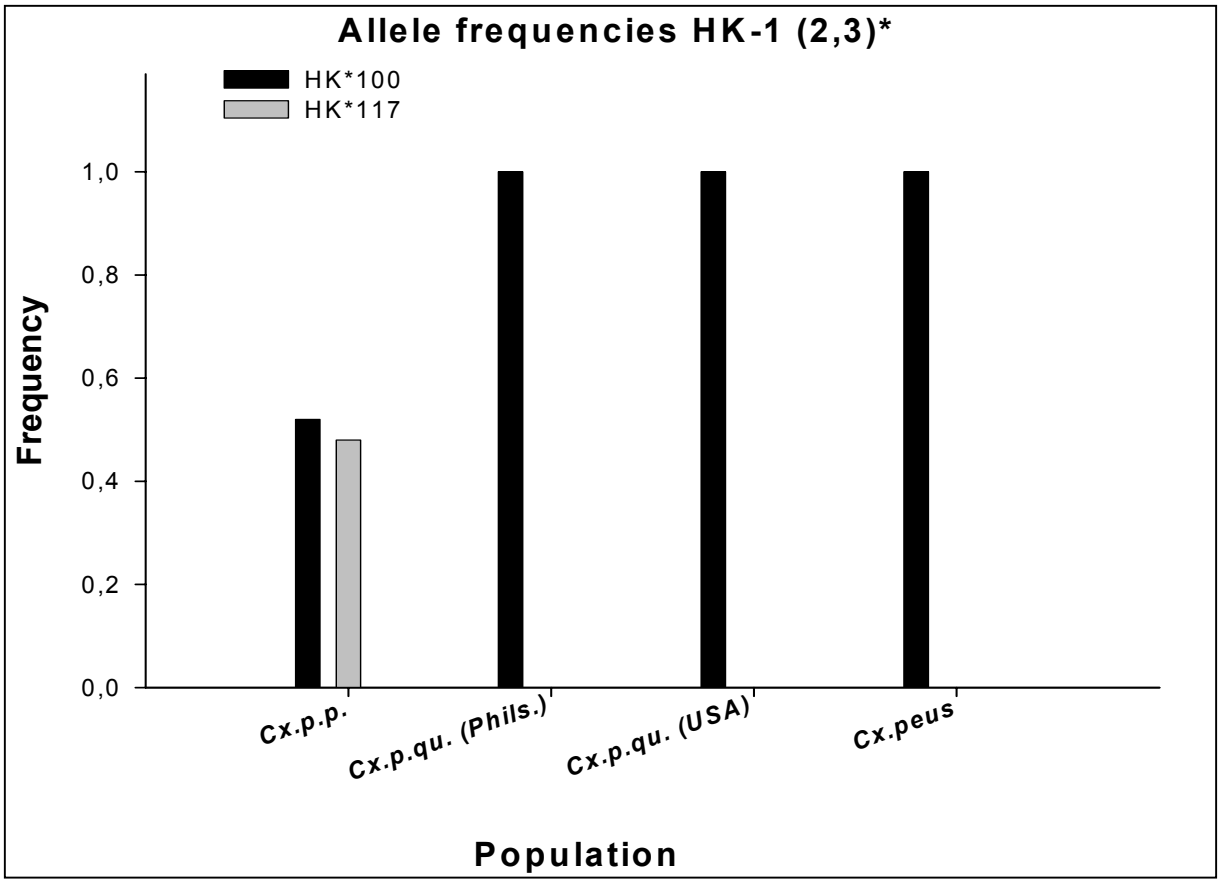
Figure 2-4A. Electrophoretic mobilities of HK in populations of *Culex*. Lanes: 1 to 16: *Cx. p. quinquefasciatus* (Phils.), 17 to 20: *Cx. p. pipiens*, 21 & 22: *Cx. peus*. **B**. Electrophoretic mobility model of Hexokinase.

Table 2-10. Absolute (Abs.) and relative (Rel.) genotype frequencies of HK-1(2,3)* in populations of *Culex* .

Locus	**HK-1(2,3)***						
Genotype	**100/100**		**100/117**		**117/117**		**Total**
Population	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	
Cx. p. pipiens	5	0.2000	16	0.6400	4	0.1600	25
Cx. p. quinquefasciatus (Phils.)	32	1.0000	0	0.0000	0	0.0000	32
Cx. p. quinquefasciatus (USA)	21	1.0000	0	0.0000	0	0.0000	21
Cx. peus	11	1.0000	0	0.0000	0	0.0000	11

Table 2-11. Absolute (Abs.) and relative (Rel.) allele frequencies as well as variances (Var.) and confidence intervals (CI) of HK-1 (2,3)* in populations of *Culex*. The confidence interval results from the relative allele frequency ± the V-value (see 2.2.9).

Allele	**HK-1 (2, 3)*100**				**HK-1 (2, 3)*117**			
		CI				**CI**		
Population	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.
Cx. p. pipiens	26	0.5200	0.0707	0.0050	24	0.4800	0.0707	0.0050
Cx. p. quinquefasciatus (Phils.)	64	1.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000
Cx. p. quinquefasciatus (USA)	42	1.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000
Cx. peus	22	1.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000



Graph 2-5. Allele frequencies of HK-1(2,3)* in populations of *Culex*.

Adenylate Kinase (AK) **E.C. 2.7.4.3**

Buffer system no. 5, Running time: 60 min., Voltage: 500 V, Ampere: 55 mA, Incubation time: 20 min.

There were two allozymic variants observed in AK. They were designated as AK*83, and AK*100 (Figure 2-5 A & B*)*. Allele AK*83 was found exclusively in *Cx. peus* with relative allele frequency of 1.0000. Allele AK*100 was observed in samples of C*ulex p. pipiens, Cx. p. quinquefasciatus* (Phils.) and *Cx. p. quinquefasciatus* (USA) with the same frequency of 1.0000 (Table 2-12 & 2-13). No heterozygosity was detected. Consequently, AK* allozymes separate *Cx. peus* from the other samples.

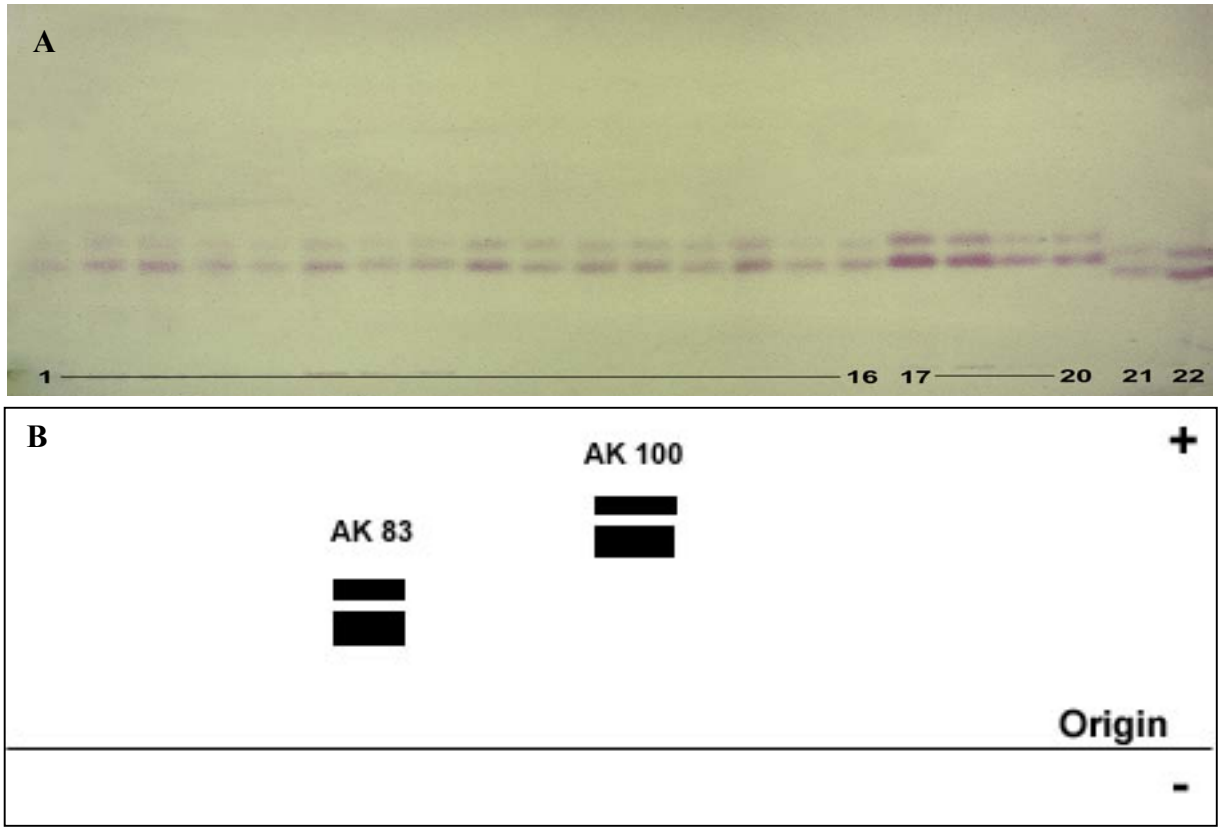


Figure 2-5A. The electrophoretic mobilities of AK in populations of *Culex.* Lanes: 1 to 16: *Cx. p. quinquefasciatus* (Phils.), 17 to 20: *Cx. p. pipiens*, 21 & 22: *Cx. peus.* **B**. A model photo of the electrophoretic mobilities of AK.

Table 2-12. Absolute (Abs.) and relative (Rel.) genotype frequencies of AK-1(2,3)* in populations of *Culex* .

Locus	**AK***						
Genotype	**83/83**		**83/100**		**100/100**		**TOTAL**
Population	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	
Cx. p. pipiens	0	0.0000	0	0.0000	37	1.0000	37
Cx. p. quinquefasciatus (Phils.)	0	0.0000	0	0.0000	16	1.0000	16
Cx. p. quinquefasciatus (USA)	0	0.0000	0	0.0000	19	1.0000	19
Cx. peus	13	1.0000	0	0.0000	0	0.0000	13

Table 2-13. Absolute (Abs.) and relative (Rel.) allele frequencies as well as variances (Var.) and confidence intervals (CI) of AK* in populations of *Culex*. The confidence interval results from the relative allele frequency ± the V-value (see 2.2.9).

Allele	**AK*83**				**AK*100**			
		CI				**CI**		
Population	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.
Cx. p. pipiens	0	0.0000	0.0000	0.0000	74	1.0000	0.0000	0.0000
Cx. p. quinquefasciatus (Phils.)	0	0.0000	0.0000	0.0000	32	1.0000	0.0000	0.0000
Cx. p. quinquefasciatus (USA)	0	0.0000	0.0000	0.0000	38	1.0000	0.0000	0.0000
Cx. peus	26	1.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000

Isomerases

Mannose phosphate-isomerase (MPI) E.C. 5.3.1.8

Buffer system no. 3, Running time: 60 min., Voltage: 500 V, Ampere: 43 mA, Incubation time: 20 min.

There were 11 electrophoretic variants depending only on a single MPI-gene locus that were observed in populations of *Culex* that were tested (Figure 2-6 A & B). These were MPI*31, 76, 100, 114, 124, 131, 138, 145, 162, 177 and 190 (Table 2-14 & 2-15). MPI*31 was observed only in *Cx. p. pipiens* with a frequency of 0.0192. Allele MPI*76 was observed in

all investigated populations of *Culex* except *Cx. peus* with frequencies of 0.1153 in *Cx. p. pipiens*, 0.1406 in *Cx. p. quinquefasciatus* (Phils.) and 0.1250 in *Cx. p. quinquefasciatus* (USA). MPI*100 was observed in three populations with frequencies of 0.211 in *Cx. p. pipiens*, 0.5469 in *Cx. p. quinquefasciatus* (Phils.) and 0.5938 in *Cx. p. quinquefasciatus* (USA). Allele MPI*114 was present in all *Culex* populations except *Cx. p. quinquefasciatus* (USA). It showed a frequency of 0.0192 in *Cx. p. pipiens* , 0.0156 in *Cx. p. quinquefasciatus* (Phils.) and 0.0714 in *Cx. peus*. Another allele is MPI*124 which was one of the commonest alleles in all four populations. This allele showed relative frequencies of 0.2500 in *Cx. p. pipiens*, 0.2812 in *Cx. p. quinquefasciatus* (Phils.), 0.0938 in *Cx. p. quinquefasciatus* (USA) and 0.1071 in *Cx. peus*. MPI*131 was only present in individuals of *Cx. p. pipiens* and *Cx. peus* with frequencies of 0.0961 and 0.0714, respectively. This allele was not found in both populations of *Cx. p. quinquefasciatus* (Phils.) and *Cx. p. quinquefasciatus* (USA*)*. Allele MPI*138 was observed in both populations of *Cx. p. pipiens* and *Cx. p. quinquefasciatus* (USA) with relative frequencies of 0.0192 and 0.0625 respectively. This allele was absent in both populations of *Cx. peus and Cx. p. quinquefasciatus* (Phils.). Allele MPI*145 was observed in three populations with frequencies of 0.0769 in *Cx. p. pipiens*, 0.1250 in *Cx. p. quinquefasciatus* (USA) and 0.4286 in *Cx. peus*. This allele was absent in *Cx. p. quinquefasciatus* (Phils.). MPI*162 was observed in three populations with frequencies of 0.1154 in *Cx. p. pipiens*, 0.0156 in *Cx. p. quinquefasciatus* (Phils.) and 0.1429 in *Cx. peus*, respectively. This allele was not observed in samples of *Cx. p. quinquefasciatus* (USA). Allele MPI*177 was present in both populations of *Cx. p. pipiens* and *Cx. peus* with relative frequencies of 0.0385 and 0.1786, respectively. This allele was absent in both populations of *Cx. p. quinquefasciatus* (Phils.) and *Cx. p. quinquefasciatus* (USA). The most mobile MPI allozyme represented the allele MPI*190 which was found only in *Cx. p. pipiens* with relative allele frequency of 0.0385.

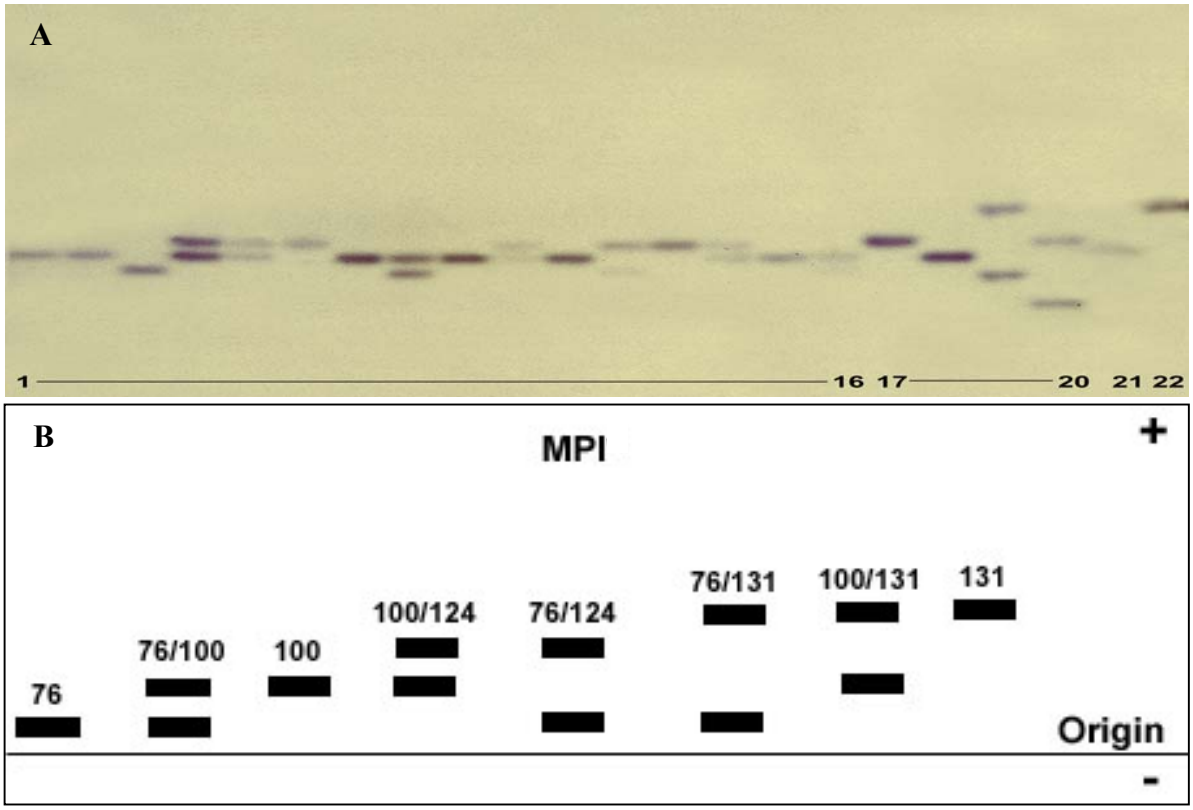


Figure 2-6A. Electrophoretic mobilities of MPI in populations of *Culex*. Lanes:1 to 16: *Cx. p. quinquefasciatus* (Phils.), 17 to 20: *Cx .p. pipiens*, 21 & 22: *Cx. peus*. **B**. A model picture of the electrophoretic mobilities of MPI.

Table 2-14. Absolute (Abs.) and relative (Rel.) allele frequencies of MPI* in populations of *Culex.*

Locus	**MPI***									
Genotype	**31/131**		**76/76**		**76/100**		**76/124**		**76/138**	
Population	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.
Cx. p. pipiens	1	0.0385	1	0.0385	0	0.0000	1	0.0385	0	0.0000
Cx. p. quinquefasciatus (Phils.)	0	0.0000	1	0.0313	5	0.1563	2	0.0625	0	0.0000
Cx. p. quinquefasciatus (USA)	0	0.0000	0	0.0000	3	0.1875	0	0.0000	1	0.0625
Cx. peus	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000

Locus	MPI*									
Genotype	**76/162**		**76/177**		**100/100**		**100/114**		**100/124**	
Population	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.
Cx. p. pipiens	1	0.0385	2	0.0769	4	0.1538	1	0.0385	1	0.0385
Cx. p. quinquefasciatus (Phils.)	0	0.0000	0	0.0000	10	0.3125	1	0.0313	8	0.2500
Cx. p. quinquefasciatus (USA)	0	0.0000	0	0.0000	4	0.2500	0	0.0000	3	0.1875
Cx. peus	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000

Locus	MPI*									
Genotype	**100/138**		**100/145**		**100/162**		**114/145**		**114/177**	
Population	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.
Cx. p. pipiens	0	0.0000	0	0.0000	1	0.0385	0	0.0000	0	0.0000
Cx. p. quinquefasciatus (Phils.)	0	0.0000	0	0.0000	1	0.0313	0	0.0000	0	0.0000
Cx. p. quinquefasciatus (USA)	1	0.0625	4	0.2500	0	0.0000	0	0.0000	0	0.0000
Cx. peus	0	0.0000	0	0.0000	1	0.0714	1	0.0714	1	0.0714

Locus	MPI*									
Genotype	**124/124**		**124/145**		**124/162**		**124/177**		**131/131**	
Population	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.
Cx. p. pipiens	3	0.1154	1	0.0385	4	0.1538	0	0.0000	2	0.0769
Cx. p. quinquefasciatus (Phils.)	4	0.1250	0	0.0000	0	0.0000	0	0.0000	0	0.0000
Cx. p. quinquefasciatus (USA)	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000
Cx. peus	1	0.0714	0	0.0000	0	0.0000	1	0.0714	0	0.0000

Locus	MPI*									
Genotype	**131/145**		**131/162**		**138/190**		**145/145**		**145/162**	
Population	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.
Cx. p. piens	0	0.0000	0	0.0000	1	0.0385	1	0.0385	0	0.0000
Cx. p. quinquefasciatus (Phils.)	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000
Cx. p. quinquefasciatus (USA)	0	0.0000	0	0.0000	0	0.0000	0	0.0000	0	0.0000
Cx. peus	1	0.0714	1	0.0714	0	0.0000	3	0.2143	1	0.0714

Locus	MPI*						
Genotype	**145/177**		**145/190**		**162/162**		**Total**
Population	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	
Cx. p. pipiens	0	0.0000	1	0.0385	0	0.0000	26
Cx. p. quinquefasciatus (Phils.)	0	0.0000	0	0.0000	0	0.0000	32
Cx. p. quinquefasciatus (USA)	0	0.0000	0	0.0000	0	0.0000	16
Cx. peus	3	0.2143	0	0.0000	1	0.0714	14

Table 2-15. Absolute (Abs.) and relative (Rel.) allele frequencies as well as variances (Var.) and confidence intervals (CI) of MPI* in populations of *Culex*. The confidence interval results from the relative allele frequency ± the V-value (see 2.2.9).

Allele	**MPI*31**				**MPI*76**				**MPI*100**			
		CI				**CI**				**CI**		
Population	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.
Cx. p. pipiens	1	0.0192	0.0200	0.0004	6	0.1153	0.0447	0.0020	11	0.2115	0.0566	0.0032
Cx. p. qu. (Phils.)*	0	0.0000	0.0000	0.0000	9	0.1406	0.0436	0.0019	35	0.5469	0.0625	0.0039
Cx. p. qu. (USA)*	0	0.0000	0.0000	0.0000	4	0.1250	0.0583	0.0034	19	0.5938	0.0866	0.0075
Cx. peus	0	0.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000

Allele	**MPI*114**				**MPI*124**				**MPI*131**			
		CI				**CI**				**CI**		
Population	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.
Cx. p. pipiens	1	0.0192	0.0200	0.0004	13	0.2500	0.0600	0.0036	5	0.0961	0.0412	0.0017
Cx. p. qu. (Phils.)*	1	0.0156	0.0141	0.0002	18	0.2812	0.0566	0.0032	0	0.0000	0.0000	0.0000
Cx. p. qu. (USA)*	0	0.0000	0.0000	0.0000	3	0.0938	0.0520	0.0027	0	0.0000	0.0000	0.0000
Cx. peus	2	0.0714	0.0490	0.0024	3	0.1071	0.0583	0.0034	2	0.0714	0.0490	0.0024

Allele	**MPI*138**				**MPI*145**				**MPI*162**			
		CI				**CI**				**CI**		
Population	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.
Cx. p. pipiens	1	0.0192	0.0200	0.0004	4	0.0769	0.0374	0.0014	6	0.1154	0.0447	0.0020
Cx. p. qu. (Phils.)*	0	0.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000	1	0.0156	0.0141	0.0002
Cx. p. qu. (USA)*	2	0.0625	0.0424	0.0018	4	0.1250	0.0583	0.0034	0	0.0000	0.0000	0.0000
Cx. peus	0	0.0000	0.0000	0.0000	12	0.4286	0.0932	0.0087	4	0.1429	0.0663	0.0044

Allele	MPI*177				MPI*190				Total
		CI				CI			
Population	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.	
Cx. p. pipiens	2	0.0385	0.0264	0.0007	2	0.0385	0.0264	0.0007	52
Cx. p. qu. (Phils.)*	0	0.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000	64
Cx. p. qu. (USA)*	0	0.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000	32
Cx. peus	5	0.1786	0.0721	0.0052	0	0.0000	0.0000	0.0000	28

***** *qu. = quinquefasciatus*

Glucose-6-phosphate-isomerase (GPI) E.C. 5.3.1.9

Buffer system no. 4, Running time: 66 min., Voltage: 450 V, Ampere: 96 mA, Incubation time: 20 min.

Two loci were observed, but only GPI of one locus showed satisfying enzymatic activity in the gels. Consequently, only one valid locus was incorporated in this result (Fig. 2-7 A & B). Three alleles were detected in populations of *Culex*. These were alleles GPI*75, GPI*100, GPI*120. Allele GPI*120 was observed only in *Cx. peus* with a frequency of 1.0000. GPI*75 was observed in both *Cx. p. pipiens* and *Cx. p. quinquefasciatus* (USA) with relative frequencies of 0.2174 and 0.3810, respectively. The most common allele (GPI*100) was observed in three populations with frequencies of 0.7826 in *Cx. p. pipiens*, 1.0000 in *Cx. p. quinquefasciatus* (Phils.) and 0.6190 in *Cx. p. quinquefasciatus* (USA), respectively. This allele was not present in *Cx. peus* (Table 2-16, 2-17 & Graph 2-6).

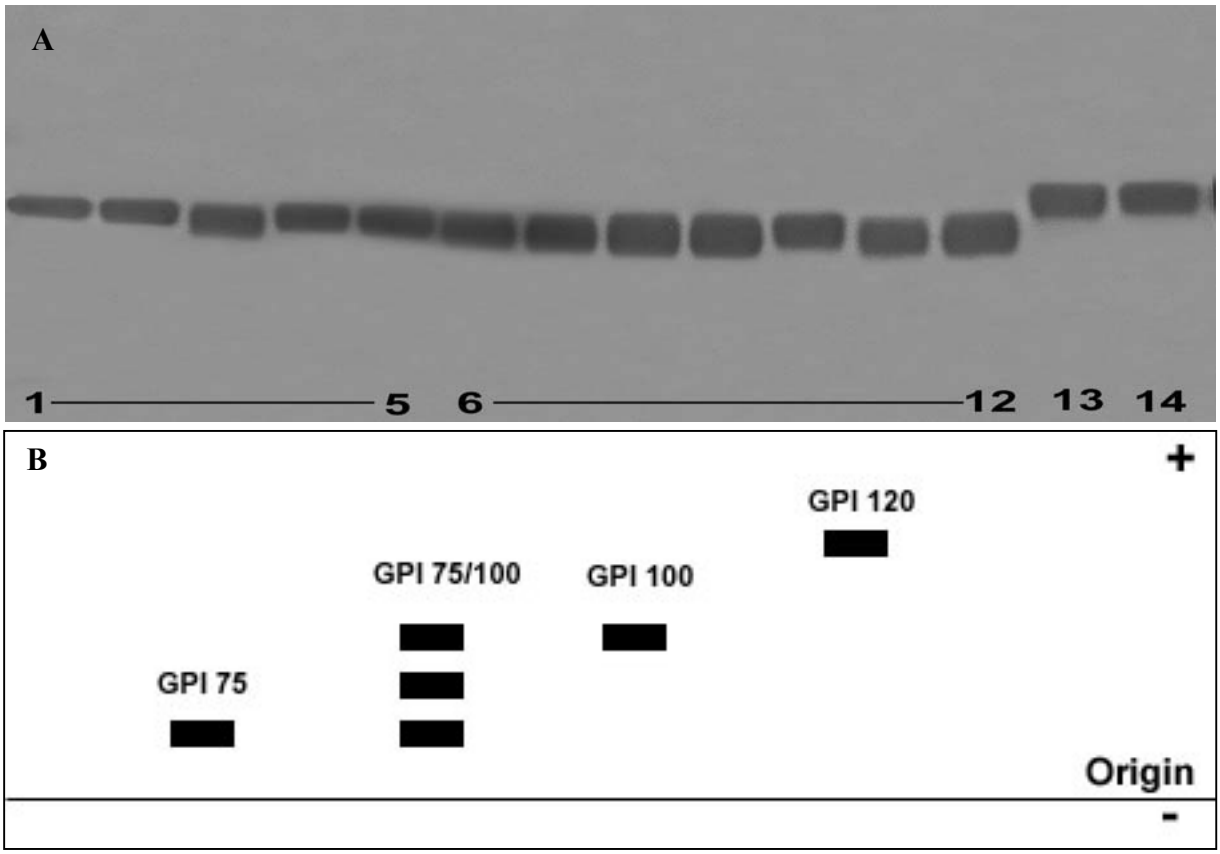


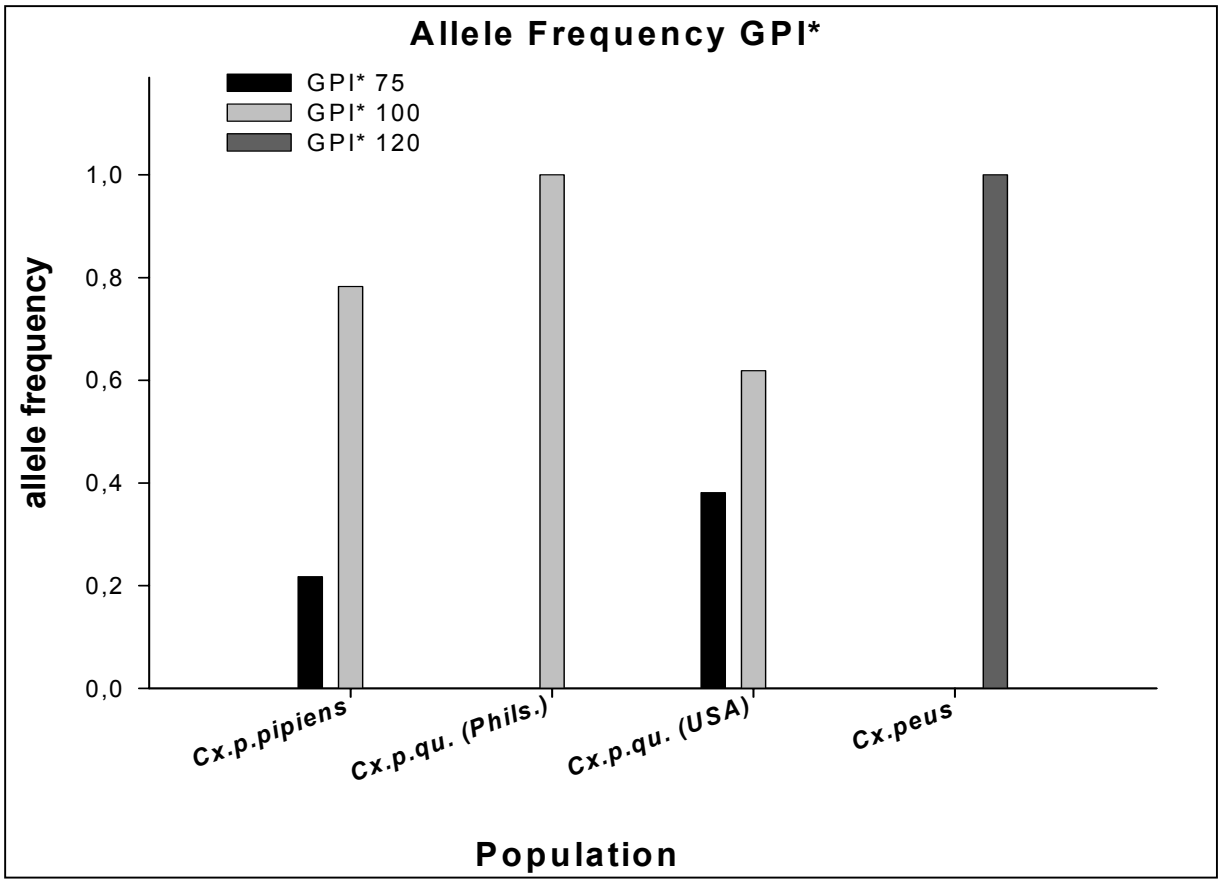
Figure 2-7A. Electrophoretic mobilities of GPI in populations of *Culex*. Lanes: 1 to 5: *Cx. p. pipiens*, 6 to 12: *Cx. p. quinquefasciatus* (USA), 13 & 14: *Cx. peus*. **B**. A model photo of the electrophoretic mobilities of GPI**.**

Table 2-16. Absolute (Abs.) and relative (Rel.) genotype frequencies at the GPI gene locus in populations of *Culex.*

Locus	**GPI***								
Genotype	**75/75**		**75/100**		**100/100**		**120/120**		**Total**
Population	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	
Cx. p. pipiens	2	0.0869	6	0.2609	15	0.6522	0	0.0000	23
Cx. p. quinquefasciatus (Phils.)	0	0.0000	0	0.0000	32	1.0000	0	0.0000	32
Cx. p. quinquefasciatus (USA)	0	0.0000	16	0.7619	5	0.2381	0	0.0000	21
Cx. peus	0	0.0000	0	0.0000	0	0.0000	13	1.0000	13

Table 2-17. Absolute (Abs.) and relative (Rel.) allele frequencies as well as variances (Var.) and confidence intervals (CI) of GPI* in populations of *Culex*. The confidence interval results from the relative allele frequency ± the V-value (see 2.2.9).

Allele	**GPI*75**				**GPI*100**				**GPI*120**			
		CI				**CI**				**CI**		
Population	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.
Cx. p. pipiens	10	0.2174	0.0608	0.0037	36	0.7826	0.0608	0.0037	0	0.0000	0.0000	0.0000
Cx. p. quinquefasciatus (Phils.)	0	0.0000	0.0000	0.0000	64	1.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000
Cx. p. quinquefasciatus (USA)	16	0.3810	0.1497	0.0056	26	0.6190	0.1497	0.0056	0	0.0000	0.0000	0.0000
Cx. peus	0	0.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000	26	1.0000	0.0000	0.0000



Graph 2-6. The distribution of allele frequencies of GPI* in populations of *Culex*.

Phosphoglucomutase (PGM) E.C. 5.4.2.2

Buffer system no. 4, Running time: 66 min., Voltage: 450 V, Ampere: 85 mA, Incubation time: 10 min.

In some culicine genera, every single PGM allele determines a two-band electrophoretic pattern with the faster moving band relatively the weaker of the two (Bullini & Coluzzi, 1972b). This pattern possibly involving intermediate steps with the more anodal band having a weak enzymatic activity or might also be modified by an increase in the size of the faster band which stains deeply, while the slower band becomes weaker. These modifications can lead to the mis-interpretations as they can imply heterozygotes or additional alleles.

There were four electrophoretic variants observed representing four different alleles (Figure 2-8A & B). These alleles were: PGM*58, PGM*100, PGM*150 and PGM*183. Allele PGM*58 was observed in both *Cx. p. pipiens* and *Cx. p. quinquefasciatus* (Phils.) with a relative frequency of 0.0800 and 0.0781, respectively. This rare allele was not observed in both populations of *Cx. p. quinquefasciatus* (USA) and *Cx. peus*. PGM*100 is the commonest allele and was observed in all populations with frequencies of 0.7200 in *Cx. p. pipiens*, 0.7031 in *Cx. p. quinquefasciatus* (Phils.), 1.0000 in *Cx. p. quinquefasciatus* (USA) and 0.9286 in *Cx. peus*. Allele PGM*150 was observed in three populations with frequency of 0.2000 in *Cx. p. pipiens*, 0.2031 in *Cx. p. quinquefasciatus* (Phils.) and 0.0714 in *Cx. peus*. This allele was absent in *Cx. p. quinquefasciatus* (USA). PGM*183 was observed only in *Cx. p. quinquefasciatus* (Phils.). The distribution of the PGM alleles in each population is presented in Table 2-18, 2-19 and Graph 2-7.

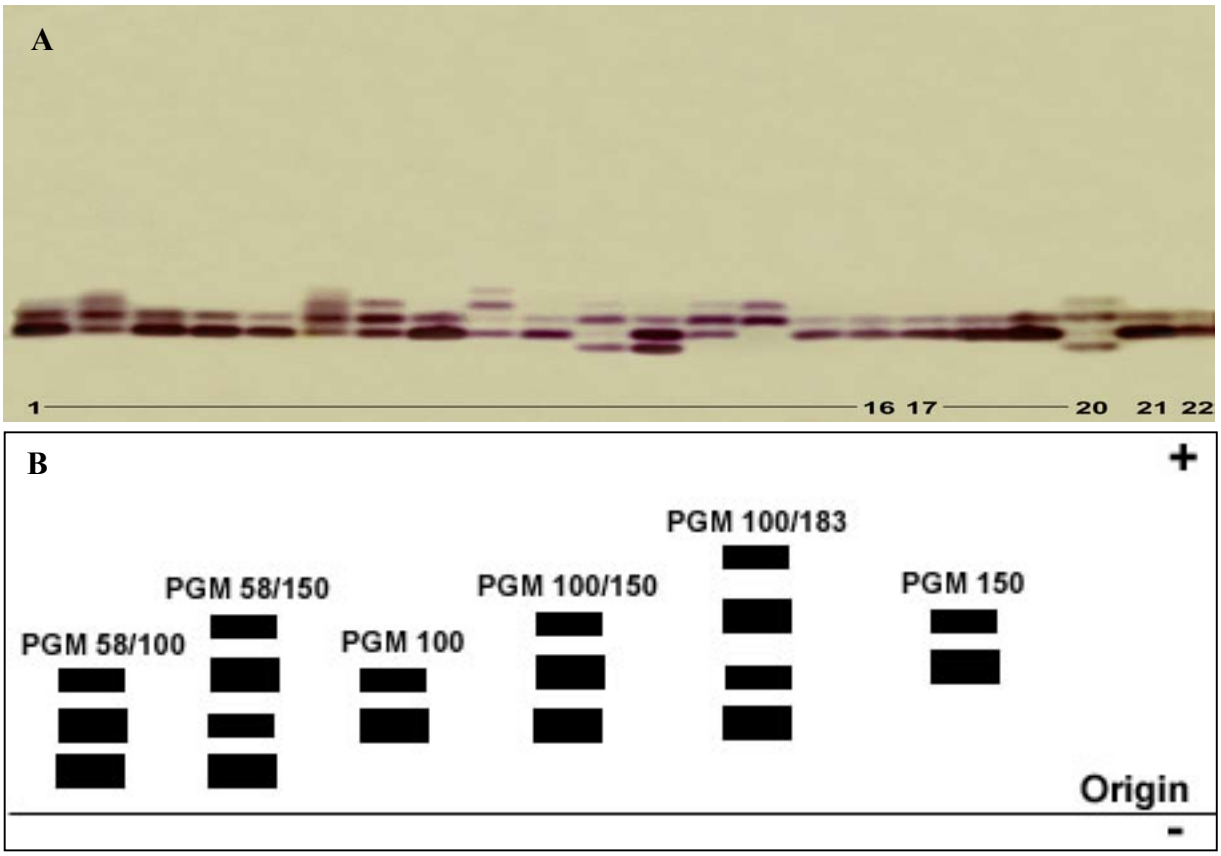


Figure 2-8A. Electrophoretic mobilities of PGM in populations of *Culex.* Lanes: 1 to 16: *Cx. p. quinquefasciatus* (Phils.), 17 to 20: *Cx. p. pipiens*, 21 & 22: *Cx. peus.* **B**. A model photo of the electrophoretic mobilities of PGM.

Table 2-18. Absolute (Abs.) and relative (Rel.) genotype frequencies of PGM* in populations of *Culex*.

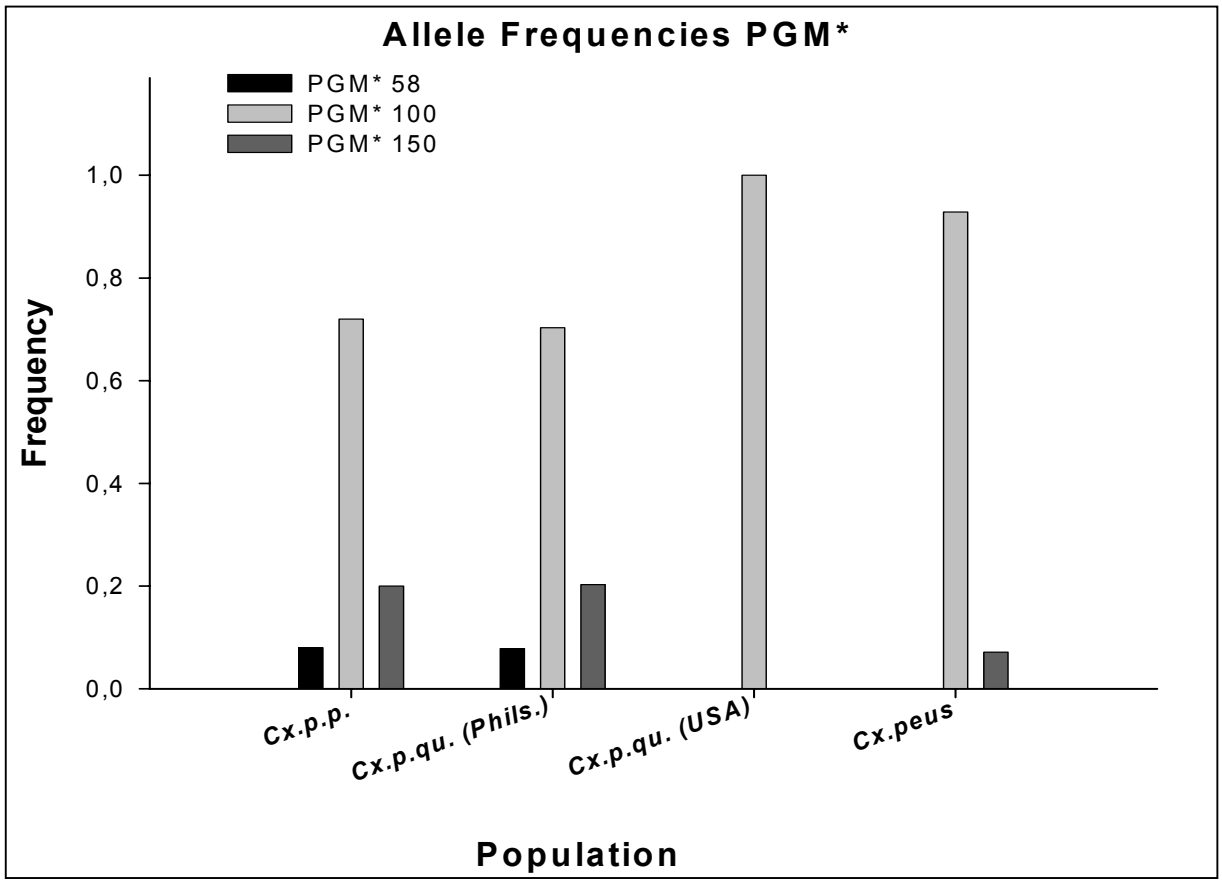
Locus	**PGM***												
Genotype	**58/100**		**58/150**		**100/100**		**100/150**		**100/183**		**150/150**		**Total**
Population	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	Abs.	Rel.	
Cx. p. pipiens	3	0.1200	1	0.0400	12	0.4800	9	0.3600	0	0.0000	0	0.0000	25
Cx. p. qu. (Phils.)*	3	0.0938	2	0.0625	17	0.5313	7	0.2188	1	0.0313	2	0.0625	32
Cx. p. qu. (USA)*	0	0.0000	0	0.0000	21	1.0000	0	0.0000	0	0.0000	0	0.0000	21
Cx. peus	0	0.0000	0	0.0000	6	0.8571	1	0.1429	0	0.0000	0	0.0000	7

* *qu. = quinquefasciatus*

Table 2-19. Absolute (Abs.) and relative (Rel.) allele frequencies as well as variances (Var.) and confidence intervals (CI) of PGM* in populations of *Culex*. The confidence interval results from the relative allele frequency ± the V-value (see 2.2.9).

Allele	**PGM*58**				**PGM*100**			
		CI				**CI**		
Population	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.
Cx. p. pipiens	4	0.0800	0.0387	0.0015	36	0.7200	0.0632	0.0040
Cx. p. quinque-fasciatus (Phils.)	5	0.0781	0.0332	0.0011	45	0.7031	0.0574	0.0033
Cx. p. quinque-fasciatus (USA)	0	0.0000	0.0000	0.0000	42	1.0000	0.0000	0.0000
Cx. peus	0	0.0000	0.0000	0.0000	13	0.9286	0.0686	0.0047

Allele	**PGM*150**				**PGM*189**			
		CI				**CI**		
Population	Abs.	Rel.	V(±)	Var.	Abs.	Rel.	V(±)	Var.
Cx. p. pipiens	10	0.200	0.0566	0.0032	0	0.0000	0.0000	0.0000
Cx. p. quinque-fasciatus (Phils.)	13	0.2031	0.0500	0.0025	1	0.0156	0.0141	0.0002
Cx. p. quinque-fasciatus (USA)	0	0.0000	0.0000	0.0000	0	0.0000	0.0000	0.0000
Cx. peus	1	0.0714	0.0686	0.0047	1	0.0000	0.0000	0.0000



Graph 2-7. Allele frequencies of PGM-2* in populations of *Culex*.

2.3.2. Degrees of heterozygosity and polymorphism of *Culex* populations

To evaluate the genetic variability of populations, the degrees of heterozygosity and polymorphism were calculated. The basis of this calculation were 12 presumptive enzyme gene loci. Concerning the uncertain genetic background of the hexokinase enzyme system, which is encoded either by three genetic loci (HK-1*, HK-2*, HK-3*), or only by one locus, the values were calculated only on the basis of 10 loci including only one HK-locus (Table 2-20A to D).

In the population of *Cx. p. pipiens* loci MDH_{NAD}-1*, MDH_{NADP}*, IDH-1* and AK* were all monomorphic while loci MDH_{NAD}-2*, IDH-2*, HK-1(2,3)*, MPI*, GPI* and PGM* were all polymorphic (Table 2-20A). In the population of *Cx. p. quinquefasciatus* (Phils.), MDH_{NAD}-1*, MDH_{NAD}-2*, MDH_{NADP}*, IDH-1*, HK-1(2,3)*, AK* and GPI* were all monomorphic while loci IDH-2*, MPI* and PGM* were polymorphic (Table 2-20B). All enzyme loci from the population of *Cx. p. quinquefasciatus* (USA) were monomorphic except GPI* and PGM* that were polymorphic (Table 2-20C). MDH_{NAD}-1*, MDH_{NAD}-2*, IDH-1*, HK-1(2,3)*, AK*

and GPI* were monomorphic while MDH_{NADP}*, IDH-2*, MPI* and PGM* were polymorphic in *Cx. peus* (Table 2-20D).

MDH_{NAD}-2* in population of *Cx. p. pipiens* as well as IDH-2* in *Cx. p. quinquefasciatus* (Phils.) showed a low level of polymorphism (Table 2-20A & B). A higher level of polymorphism was observed at HK-1 (2, 3)*, MPI*, PGM*, MPI* and GPI* (Table 2-20A to D). The summary of the polymorphism as well as the heterozygosity are summarized in Table 2-21.

The populations of *Culex* were in Hardy-Weinberg equilibrium, except the population of *Cx. p. quinquefasciatus* from the USA (Appendix 2-1 to 2-3). This population showed significant deviations from Hardy-Weinberg equilibrium at locus GPI* because of the deficiency of heterozygotes as shown by the result of chi square adaption test (Appendix 2-3). Furthermore, the deviation from Hardy-Weinberg equilibrium was not calculated in the population of *Cx. peus* because of the small amount of samples being used in the study.

Table 2-20A. Allozyme variability in 10 gene loci of *Culex p. pipiens*.

Enzyme locus	Allele	Frequency	Polymorphic (p) / monomorphic (m)	Degree of heterozygosity
MDH_{NAD}-1*	100	1.0000	m	
MDH_{NAD}-2*	100	0.9500	p	0.0500
	124	0.0500		
MDH_{NADP}*	100	1.0000	m	
IDH-1*	100	1.0000	m	
IDH-2*	84	0.0946	p	0.1351
	100	0.9054		
HK-1(2,3) *	100	0.5200	p	0.6400
	117	0.4800		
AK*	100	1.0000	m	
MPI*	31	0.0192	p	0.5769
	76	0.1153		
	100	0.2115		
	114	0.0192		
	124	0.2500		
	131	0.0961		
	138	0.0190		
	145	0.0769		

	162	0.0156		
	177	0.0385		
	190	0.0385		
GPI*	75	0.2174	p	0.2609
	100	0.7826		
PGM*	58	0.0800	p	0.5200
	100	0.7200		
	150	0.2000		
			$P_{\varnothing}$ = 0.6000	$H\varnothing$ = 0.2183

Table 2-20B. Allozyme variability in 10 gene loci of *Culex p. quinquefasciatus* (Phils.).

Enzyme locus	Allele	Frequency	Polymorphic (p) / monomorphic (m)	Degree of heterozygosity
MDH $_{NAD}$-1*	100	1.0000	m	
MDH $_{NAD}$-2*	100	1.0000	m	
MDH $_{NADP}$*	100	1.0000	m	
IDH-1*	100	1.0000	m	
IDH-2*	84	0.0313	p	0.0938
	72	0.0781		
	100	0.8906		
HK-1(2,3) *	64	1.0000	m	
AK*	100	1.0000	m	
MPI*	76	0.1406	p	0.5313
	100	0.5469		
	114	0.0156		
	124	0.2812		
	162	0.0156		
GPI*	100	1.0000	m	
PGM*	58	0.0781	p	0.4063
	100	0.7031		
	150	0.2031		
	183	0.0156		
			$P_{\varnothing}$ = 0.3000	$H_{\varnothing}$ = 0.1292

Table 2-20C. Allozyme variabilty in 10 gene loci of *Cx. p. quinquefasciatus* (USA).

Enzyme locus	Allele	Frequency	Polymorphic (p) / monomorphic (m)	Degree of heterozygosity
MDH_{NAD}-1*	100	1.0000	m	0.0000
MDH_{NAD}-2*	100	1.0000	m	0.0000

MDH_{NADP}*	100	1.0000	m	0.0000
IDH-1∗	100	1.0000	m	0.0000
IDH-2∗	100	1.0000	m	0.0000
HK-1(2,3) ∗	100	1.0000	m	0.0000
AK∗	100	1.0000	m	0.0000
MPI∗	76	0.1250	p	0.7500
	100	0.5938		
	124	0.0938		
	138	0.0625		
	145	0.1250		
GPI∗	75	0.3810	p	0.7619
	100	0.6190		
PGM∗	100	1.0000	m	0.0000
			$P_{\varnothing}$= 0.2000	**$H_{\varnothing}$= 0.1512**

Table 2-20D. Allozyme variabilty in 10 gene loci of *Cx. peus.*

Enzyme locus	**Allele**	**Frequency**	**Polymorphic (p) / monomorphic (m)**	**Degree of heterozygosity**
MDH_{NAD}-1*	100	1.0000	m	0.0000
MDH_{NAD}-2∗	76	1.0000	m	0.0000
MDH_{NADP}	100	0.1000	p	0.2000
	107	0.9000		
IDH-1*	140	1.0000	m	0.0000
IDH-2*	84	0.9091	p	0.1818
	100	0.0909		
HK-1(2,3) *	100	1.0000	m	0.0000
AK*	83	1.0000	m	
MPI*	114	0.0714	p	0.7143
	124	0.1071		
	131	0.0714		
	145	0.4286		
	162	0.1429		
	177	0.1786		
GPI*	120	1.0000	m	0.0000
PGM*	100	0.9286	p	0.1429
	150	0.0714		
			$P_{\varnothing}$ = 0.4000	**$H_{\varnothing}$ = 0.1239**

Table 2-21. Summary of the average polymorphism and heterozygosity in populations of *Culex*.

Population	Polymorphism	Degree of heterozygosity
Culex p. pipiens	0.6000	0.2183
Culex p. quinquefasciatus (Phils.)	0.3000	0.1292
Culex p. quinquefasciatus (USA)	0.2000	0.1512
Culex peus	0.4000	0.1239

2.3.7. Genetic identities and distances in populations of *Culex*.

The results show genetic variations among the populations of *Culex*. The population of *Culex p. pipiens* has the genetic distances of 0.0423, 0.0504 and 1.1601 to the populations of *Cx. p. quinquefasciatus* (Phils.), *Cx. p. quinquefasciatus* (USA) and *Cx. peus*, respectively (Table 2-22). In the population of *Cx. p. quinquefasciatus* (Phils.), the genetic distances of 0.0423, 0.0288 and 1.1020 were observed to the populations of *Cx. p. pipiens*, *Cx. p. quinquefasciatus* (USA) and *Cx. peus*, respectively (Table 2-22). *Cx. p. quinquefasciatus* (USA) has the genetic distances of 0.0504, 0.0288 and 1.0228 to the populations of *Cx. p. pipiens*, *Cx. p. quinquefasciatus* (Phils.) and *Cx. peus* (USA), respectively (Table 2-22).

In contrary, the population of *Culex peus* showed a high genetic distance to the populations of *Cx. p. pipiens* as well as the population of *Cx. p. quinquefasciatus* from the Philippines and USA. This is explained by the fact that the population *Cx. peus* is not a member of the Culex Pipiens Complex. It was just included in the study in order to serve as an outgroup taxon for the evaluation of the genetic differentiation between *Culex pipiens* populations.

Furthermore, the construction of the dendrogram based on the UPGMA-cluster, revealed that the populations of *Cx. p. pipiens* and *Cx. p. quinquefasciatus* from the Philippines and USA fall into one cluster, whereas the population of *Cx. peus* belong to a very distinct cluster (Fig. 2-9).

Table 2-22. Genetic distances in populations of *Culex*.

	Cx. p. pipiens	*Cx. p. quinquefasciatus* (Phils.)	*Cx. p. quinquefasciatus* (USA)	*Cx. peus*
Cx. p. pipiens	*	0.0423	0.0504	1.1601
Cx. p. quinque-fasciatus (Phils.)	0.0423	*	0.0288	1.1020
Cx. p. quinque-fasciatus (USA)	0.0504	0.0288	*	1.0228
Cx. peus	1.1601	1.1020	1.0228	*

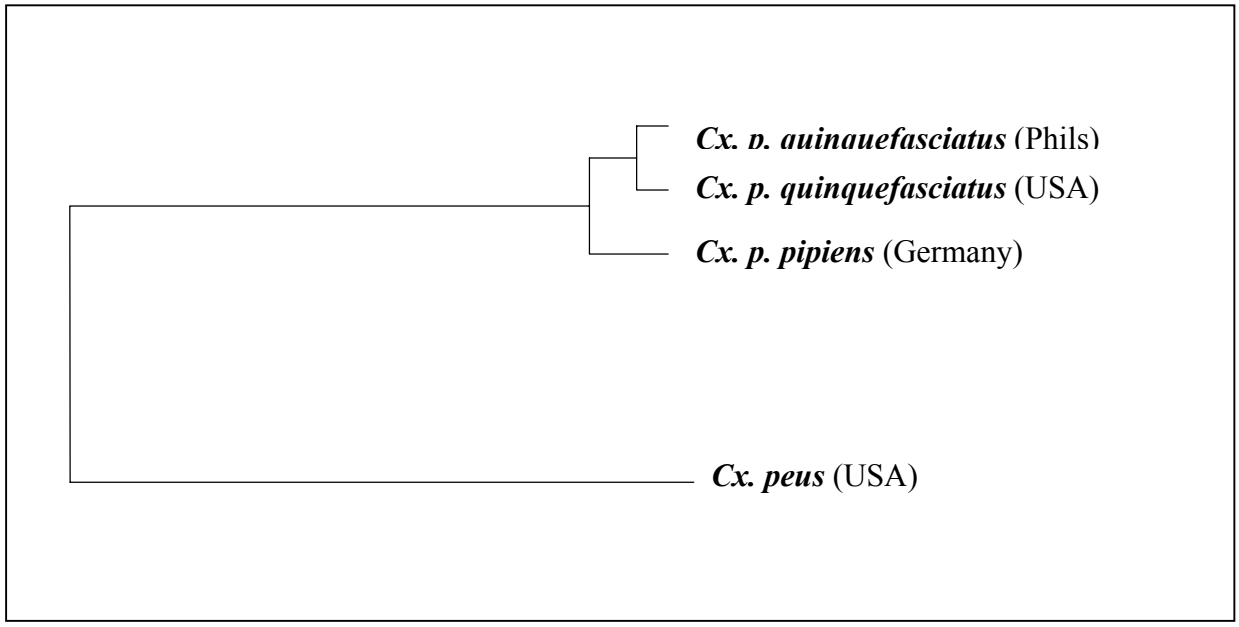


Fig. 2-9. The UPGMA-Dendrogram based on genetic distances Nei (1972) demonstrates the low genetic differentiation between the populations of Culex Pipiens Complex in contrast to *Cx. peus*.

2.4. DISCUSSION

Typical biological characters of the species and subspecies of the Culex Pipiens Complex as their widespread distribution, population sizes and the mobility of mating and host-seeking adults lead to the assumption of a high genetic polymorphism as characteristic. Their ecological flexibility may prevent frequent population breakdowns and bottleneck-effects. The mating behaviour of *Culex pipiens* seems to be rather non-selective and panmictic. Limited population density oscillation as common in mosquito populations, usually does not affect well-balanced heterozygosity, and in addition, genetic variability including even rare alleles can be maintained.

In contrast to the high intraspecific variability, these factors indicate and confirm low genetic differentiation between geographic *Culex pipiens* populations because of a continuous gene flow but low degree of random genetic drift. Theoretically, genetic differentiation between geographic populations increases due to conditions like fragmented species distribution including geographic isolation, colonization of new habitats and frequent bottleneck-effects. Both selection and random genetic drift, get enhanced by former conditions. As shown above and confirmed by the electrophoretic data, these factors seem to be represented in species like *Cx. pipiens*.

This multilocus electrophoretic study quantifies the genetic variability within and differentiation between the populations of *Culex* at 10 enzyme gene loci. The highest average degree of polymorphism (P = 0.6000) was observed in the population of *Cx. p. pipiens* whereas, *Cx. p. quinquefasiatus* from the USA had the lowest average polymorphism of P = 0.2000 (Table 2-21). Enzyme phenotypes observed in *Cx. p. pipiens* and *Cx. p. quinquefasciaus* were mostly similar to those described by Steiner and Joslyn (1979). However, the observed AK homozygous pattern indicated a one-allele-two-band situation as reported for certain other enzymes in *Cx. pipiens* (Bullini & Coluzzi, 1973). Interestingly, MPI* and PGM* were polymorphic in all populations except PGM* in *Cx. p. quinquefasciatus* from the USA which was monomorphic.

Likewise, the highest average degree of heterozygosity (H = 0.2183) was observed in the population of *Cx. p. pipiens,* whereas the lowest average heterozygosity of H = 0.1292 was observed in the population of *Cx. p. quinquefasciatus* from the Philippines excluding the outgroup species of *Cx. peus* (Table 2-21). Barr (1981) cited that *Cx. pipiens* has higher degree of heterozygosity compared with *Cx. p. quinquefasciatus* because of the limited but continuous gene flow. McDonald and Ayala (1974) pointed out that higher levels of heterozygosity were expected to occur in populations exposed to various environmental conditions, particularly temperature. This is explained by the results obtained from this study. *Cx. p. quinquefasciatus* from the Philippines and USA (California) were sampled from countries having stable climatic conditions and low variations of breeding sites. Therefore, it leads to a little selection pressure. In contrary, the population of *Cx. p. pipiens* collected from Germany was exposed to various types of environmental conditions.

The relatively low level of variability observed in the populations of *Cx. p. quinquefasciatus* from the Philippines and USA could also be induced by mode of sampling, the origin of the population (maybe offspring of only a few egg-laying females) or gene pool isolation caused by low breeding site density in the distribution area of the respective population. These assumptions correlate with those of Farid et al. (1991) concerning the genetic similarity among Egyptian populations of *Culex pipiens*. In their study, the authors found that in Egypt, the subtropical climatic conditions lead towards uniformity and therefore low levels of heterozygosity and polymorphism were observed among the mosquito individuals. Furthermore, Barr (1957) stated that hybridization occurs where the range of these two subspecies overlap and that they interbreed in captivity (Kitzmiller, 1950).

The sample populations of *Culex* were in Hardy-Weinberg equilibrium, except the population of *Cx. p. quinquefasciatus* from the USA, where significant deviation from Hardy-Weinberg equilibrium at the locus GPI*, as shown by the result of Chi square test (Appendix 2-3). The population of *Cx. peus* was not deviated according to Hardy-Weinberg equilibrium because of the small amount of samples used in the study.

The populations of *Cx. p. pipiens* (Germany) and *Cx. p. quinquefasciatus* from the Philippines and USA, showed very low genetic distances to each other reflecting either a slow differentiation process or persisting introgression and gene flow. The distance values were characteristic of those observed among local conspecific populations of other mosquito species indicating considerable gene flow even among geographically remote population (Ayala, 1975). Brust and Munstermann (1992) as well as Schutz and Eldridge (1993) found similar genetic distance values between several populations of Ochlerotatus Communis Complex species ranging between 0.000 and 0.461.

Low genetic distances are due to the nearly worldwide and non-fragmented distribution of these two sibling species with a broad hybrid zone (Urbanelli & Bullini, 1985). Maybe selection factors are influencing the considerable ecological adaptivity of *Culex pipiens* specimen, concerning breeding site quality, ability to undergo diapause and even undetermined host preferences. Furthermore, their low morphological differentiation indicates a closed phylogenetic relationship (Bullini & Coluzzi, 1978).

In contrast, the population of *Culex peus* showed a high genetic distance to the populations of *Cx. p. pipiens* as well as in *Cx. p. quinquefasciatus* from the Philippines and USA. This is explained by the fact that the population of *Cx. peus* is not a member of the Culex Pipiens Complex. It was just included in the study in order to serve as an outgroup taxon for the evaluation of the genetic differentiation between *Culex pipiens* populations. Obviously, low genetic differentiation is not a general character of the genus *Culex*, as the genetic distances between *Cx. pipiens* populations and *Cx. peus* ranged from 1.0228 to 1.1601, whereas the low distance values between the *Cx. pipiens* populations ranged from 0.0288 to 0.0504.

Furthermore, the dendrogram constructed by UPGMA-cluster analyses confirms that the populations of *Cx. p. pipiens* (Germany)and *Cx. p. quinquefasciatus* from the Philippines and USA fall into one cluster, whereas the population of *Cx. peus* belong to a very distinct cluster (Fig. 2-9).

Pryor and Daly (1991) cited that although the two sibling species may be distinct morphologically in their respective ranges, they clearly behave as subspecies in areas of contact and gene exchange in geographic isolation (hybrid zones). Lastly, the genetic affinities determined by electrophoretic data in this study, agreed with taxonomic affinities ascertained by some authors (Pryor & Daly, 1991; Farid et al., 1991; Urbanelli et al., 1995). Thus, the present electrophoretic analysis proved to be useful in studying the genetic structure of *Cx. p. pipiens* and *Cx. p. quinquefasciatus* as well as in measuring the gene flow among these sibling species (Humeres et al., 1990).

Even when no allelic differentiation of taxonomic significance between the *Cx. pipiens* subspecies was detected, this study exhibited the genetic status of a genetically and ecologically exceptional variable and adaptable species complex. Only comprehensive knowledge of this mosquito species is of considerable medical importance that enables man to reduce the negative impact on human life conditions.

3. Studies on ovaries of mosquitoes using light, transmission and scanning microscopy

3.1. INTRODUCTION

The great majority of insects including mosquitoes reproduce sexually. Within the gonads of the ovaries, gametes differentiate and mature through a sequence of events that involves cells in complex structural and physiological changes (Smith, 1968). Female mosquitoes have two symmetrically arranged ovaries which are located parallel to the alimentary canal in the abdomen. These ovaries are located between the fourth and sixth segments of the abdomen (Roth & Porter, 1964).

Mosquito ovaries are classified as meroistic type because they contain nurse cells as well as oocytes. Furthermore, they are categorized as polytrophic because groups of nurse cells are enclosed with an oocyte in each ovarian follicle. In contrast to other insects, like for instance mayflies (Ephemeroptera) and cockroaches (Blattodea), no nurse cells are present within the oocytes. This ovary is classified as telotrophic meroistic type. In these insects, the follicle cells are equipped to carry out secretion and protein synthesis (Smith, 1968).

A central chamber or calyx is present in each paired ovary. The calyx is lined by an epithelium which contains muscle filaments that are also present in the lateral oviduct. Two tracheae enter each ovary and branch into the tracheoles within the ovarioles. Each paired ovary consists of variable numbers of tubular epithelial ovarioles where the oocytes are placed in a linear sequence according to their stage of growth (Smith,1968).

The number of ovarioles ranges from 50 to 500 depending on the species, physiological stages as well as the size of the individual female (Clements, 1992). Each of the ovariole is enclosed by a sheath which consists of cells forming a thin squamous mesothelium. Proximally, the ovarian sheath forms the long suspensory ligament which inserts into the 4^{th} abdominal tergite.

The ovariole contains a germarium as well as a maturing and presumptive follicle. In the anterior part of each ovariole, are the differentiated cells of germarium through which the mitotic divisions of germ cells takes place resulting in the formation of the oocytes. In

mosquitoes, these are accompanied by other cells which later perform the task of nursing or supplying nutrients to the developing gametes. In other insects, however, special nurse cells are absent and instead the nutritional responsiblity falls upon the epithelium of the oocyte follicles (Smith, 1968). In the posterior portion of the ovariole, approaching to the oviduct, lies the vitellarium which is composed of increasingly large and advanced oocytes, each is located within a follicular sac. When the oocyte is fully mature, it passes into the oviduct. Each of the follicle contains seven nurse cells and one oocyte within the follicular epithelium and the youngest follicle cells "oogonia" lie in the distal position (Roth & Porter, 1964). The germarium, nurse cells, follicles and follicular stalks which compose each ovariole are invested by a basal lamina, which is sometimes called tunica propria (Bertram & Bird, 1961; Anderson & Spielman, 1971).

The common oviduct as well as the posterior parts of the lateral oviducts and most of the accessory glands are ectodermal in origin, whereas the real reproductive organ, the ovaries, are of mesodermal origin. The ovaries develop from a special section of splanchnic mesoderm, which is called the genital ridge. This ridge bears the germ line cells, which are sometimes segmentally separated into smaller groups.

Female mosquitoes have various sources of food during their life period. For the regular metabolism, they are feeding on flowers and fruits as a source of energy (e.g. for flying). However, for the development of eggs, they usually need a blood meal as a source of protein. The physiological and structural changes that occur after having a blood meal are still under intensive investigations. In this chapter, the ultrastructure of mosquito ovaries with and without blood meal has been studied. Furthermore, the precise knowledge on the ultrastructure of the mosquito ovaries obtained serves as a basis for the microscopic identification of *Wolbachia* sp. infection.

3.2. MATERIALS AND METHODS

Mosquito strains

The mosquitoes used in this study were composed of six different strains, namely: Culex Pipiens Complex, *Aedes aegypti, Aedes vexans, Ochlerotatus cantans*, *Ochlerotatus rusticus* and *Anopheles maculipennis*. These were collected from different breeding sites in various localities (Table 3-1).

Table 3-1. Breeding sites and localities of mosquito strains

mosquito strain	type of breeding site	locality
Cx. pipiens	stagnant canal	Wittenweier, Germany
Ae. aegypti	old tire	Cebu City, Philippines
Ae. vexans	flood plains	Speyer, Germany
Oc. cantans	swampy woodlands	Hassloch, Germany
Oc. rusticus	swampy woodlands	Hassloch, Germany
An. maculipennis s.l.	semi-permanent water body	Bobenheim-Roxheim, Germany

Collection of mosquito samples

Mosquito samples were collected during larval stage. The collection was done by scooping the mosquito larvae with a fine mesh net. The larvae were placed in a small jar bottles containing water and covered with screens or fine nets (for detailed procedure, see chapter 2.2).

3.2.1. Transmission electron microscopy

Individual mosquito samples were placed in a small petri dish with 2.5 % glutaryldehyde in 0.1 M cacodylate buffer (pH 7.4) for the fixation process. Samples were viewed under the stereoscope for sectioning. The abdomens were sectioned into three parts, namely: anterior, middle, and posterior. The most anterior and posterior part of the abdomen were discarded. Each section was placed in a small glass vial which contained 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4°C. The samples were washed three times with 0.1 M

cacodylate buffer for a period of 10 min. and then washed with 1% osmium tetroxide for 2 h (Appendix 3-1). After osmium tetroxide, the samples were washed 3 times for a period of 30 min. with 0.1 M cacodylate buffer (pH 7.4) and were washed again three times with 0.05 M malic acid buffer (pH 5.2) for a period of 30 min. The samples were transferred to 1% uranyl acetate in 0.05 M malic acid buffer (pH 5.2) for a period of at least 2 h to overnight.

After the samples were soaked in 1% uranyl acetate in 0.05 M malic acid buffer (pH 5.2) (Appendix 3-2), these were washed three times with 0.05 M malic acid buffer (pH 5.2) for a period of 15 min. Tissues were dehydrated three times per step in an increasing concentrations of ethanol, namely: 50, 70, 80, 96, & 100%, each for 10 min. (Roth & Porter, 1964).

After dehydration, tissues were infiltrated overnight with 1:1 mixture of Spurr pur and 100 % ethyl alcohol (Appendix 3-3 & 3- 4). The samples were infiltrated again in a mixture of 3:1 Spurr pur and 100% ethanol for at least 1 h and were further infiltrated with Spurr pur for a period of at least 6 h (Appendix 3-5). Tissues were embedded and polymerized at 60°C for at least 1 day.

Trimming and mounting the specimen block for sectioning

The specimen block was firmly clamped into the appropriate specimen holder after removal from the embedding mould for ultra-microtome sectioning. Prior to sectioning, the specimen block was trimmed in order to define the shape and size of the sections. This was done by using a single sided razor blade. Specimen blocks were trimmed in various shapes depending on the form of the mounted specimen.

Glass knives were used in cutting the ultra-thin sections. The glass was cleaned prior to cutting with soap water and dried with a smooth cotton cloth. Upon handling the glass, proper care was taken into consideration in order to avoid finger prints on the narrow sides of the strip where the knife edges were formed. The glass plate was broken into strips or large squares in order to obtain glass knives.

Much care was focused in handling the knives because a good knife will quickly become a bad one upon careless handling. The glass knife was blunted as each section was cut and this

affected the thickness of the section that was subsequently cut. When the quality of the section deteriorated to less than that was required, a new part of the knife edge was used.

Semi-thin sections

Knowledge concerning the structure of the specimen as seen in light microscope was very essential before viewing the sections under electron microscopy. Semi-thin sections were very useful in the selection of suitable ultra-thin sections especially if there were several specimens. Additionally, semi-thin sections were important because it showed an evaluation in the preparation technique, such as inadequate penetration of the embedding medium into the denser parts of the specimen. Furthermore, semi-thin sections were done until the region of the ovary of mosquito was observed.

The optimum semi-thin section thickness for light microscopy is between 0.2 and 2 μm. These sections were cut using glass knives in a microtome. The sections were collected carefully from the glass knife without folding the knife edge and placed onto the surface of a glass slide with distilled water. This was done by using a small, fine glass pipette. Excess water in the glass slide was drained off by allowing the slide to dried into the hot plate at 60-80°C. After drying, Richardson's stain was added to the slide that contained the sections. When the stain had dried a little, it was washed immediately with distilled water and dried again on the hot plate. Semi-thin sections were viewed under light microscope.

Ultra-thin sections

During the process of adjusting the knife, the specimen holder was attached to the specimen arm before fixing the knife to prevent damage of the knife, although final adjustment of the specimen was still made later when the knife was in position. The specimen block was securely clamped in its holder to prevent any movement during sectioning.

Distilled water was filled up into the edge glass of the knife until the knife edge was completely wet. A droplet of distilled water was continuously added until an area of white reflected light was seen on the liquid surface.

The first contact between the knife and the specimen block was very important as both can be damaged if care was not taken. Proper care was observed that there was only a few micrometers gap between the knife and the specimen block. During the start of sectioning, the specimen arm was either operated manually or under automatic control and the knife advanced in small increments of approximately 1 μm between each stroke. Once the contact had been made, the sectioning was allowed to continue until sufficient sections were obtained or until the sections became already erratic. The ultra-thin sections were collected from the glass knife by using a fine glass pipette.

Contrasting the ultra-thin sections

Ultra-thin sections were contrasted two times using uranyl acetate and lead citrate for a period of three min. and were washed with distilled water. Washing was done by dipping the sections 10 times in four small bottles containing distilled water.

3.2.2. Scanning microscopy

The whole individuals of male and female mosquitoes were fixed for scanning microscopy. Fixing was done by using a primary cold fixative of 2.5% glutaradehyde in 0.1 M cacodylate buffer (pH 7.4) for a period of 2 h. This was followed by a buffer rinse of 0.1 M cacodylate three times for a period of 30 min. Secondary fixation was done by using 1% osmium tetroxide for two h. Samples were rinsed again 3 times with 0.1 M cacodylate buffer for a period of 30 min.

Following the appropriate fixation method, the mosquito ovaries were dehydrated for 30 min. through a graded series of acetone (50%, 70%, 80%, 90% & 100%). Dehydrated specimens were transferred from absolute acetone to a critical point drying apparatus. Critical point method is a common drying procedure using liquid carbon dioxide. The dehydrated specimens were placed in the specimen drying chamber of the apparatus. Enough absolute acetone was poured into the chamber to prevent air drying prior to critical point drying. The cover of the specimen chamber is secured and liquid carbon dioxide is added to the chamber from the tank. Acetone and carbon dioxide were mixed freely and the process was repeated eight times until carbon dioxide was only remained in the samples. The critical temperature of carbon dioxide is 31°C and 73.8 bar until the samples were totally dried.

Mosquito specimens dried by the critical method were mounted on stubs. Specimen stubs were coated with a thin layer of silver conducting paint and were allowed to dried in the petri dishes for overnight. The samples were then spotted with gold spotted SCD005 Baltec and were photographed using SEM 505 Philips.

3.2.3. Light microscopy

The abdomens of the female mosquitoes (from 2nd to 6th segments) which contained the paired ovary were detached from the rest of the body. Samples were fixed in bouin's fluid for a period of 24 h and were dehydrated through a graded series of ethanol (70%, 80%, 90%, 96% & 100%). Samples were kept three times in a mixture of methylbenzoate (intermedium alcohol/paraplast) before they were embedded two times in paraplast liquid and mounted by using paraplast. By means of a microtome, sections of 6 µm thick were cut.

The sections were stained by three different kinds of stains, namely; haematoxylin eosin and two different special stains from Goldner and Azan. After staining, the sections were mounted permanently by using depex as a mounting medium and covered permanently with glass slips and photographed under light microscope.

3.3. RESULTS

3.3.1. Transmission electron microscopy

The ultra-structure of mosquito ovaries without blood meal

The following transmission electron micrographs presented below are observed based on the microscopic study on the ovaries without blood meal.

Each of the ovary of mosquito is compose of a reproductive units called ovarian follicles or epithelial follicles (Fig. 3-1A). There are little cytoplasm that are present in the epithelial cells and the cells are cuboidal with large nuclei (Fig. 3-1A). Beneath the epithelial follicles lies the oocyte having no distinct shape of the nucleus (Fig. 3-1A). Mitochondria is present within the oocyte and several unoriented microvilli are also observed in the epithelial follicle (Fig. 3-

1B). If the mosquito ovary is unfed with blood, the cells of the ovarian follicles have no continuous epithelium but instead they possess isolated cells with gaps between them. These epithelial follicles are in close proximity and joined by each other at the apical margins by desmosomes (Fig. 3-1 B & C).

The basal surface of the ovarian follicles lies on a basement membrane or basal lamina (Fig. 3-2A). The nuclei of an ovarian follicles are seen to possess a distinct polymorphic nucleolus which is enveloped by a prominent granular chromosomal mass. Several tracheoles are seen outside the basement membrane of the ovarian follicles (Fig. 3-2B).

Mitochondria are seen very frequently in the ovarian follicles which possess well ordered, parallel arrays of cristae (Fig. 3-3A). Other major inclusion that is seen in the ovarian follicles are the lysosomes (Fig. 3-3B).

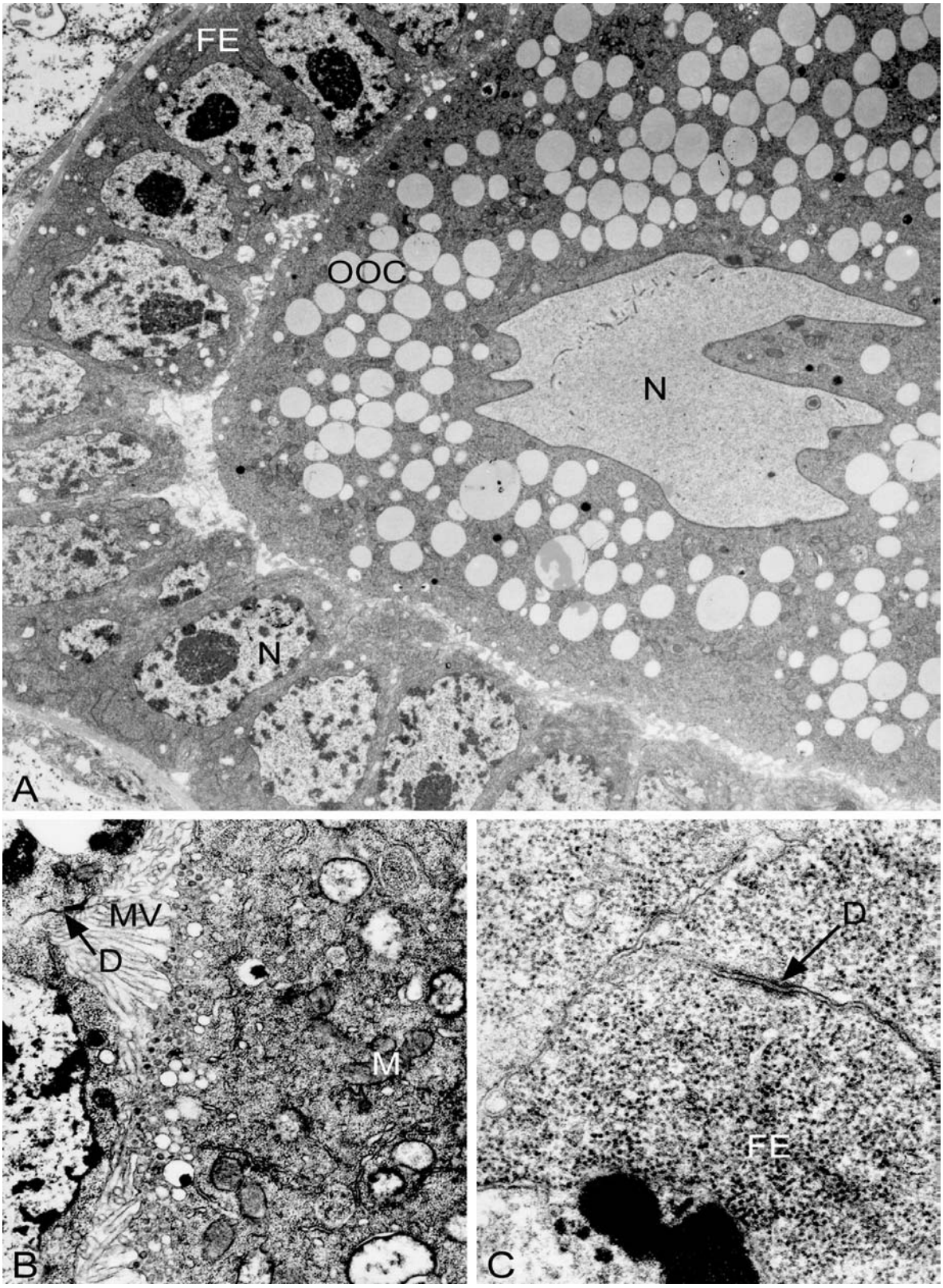


Fig. 3-1A. Transmission electron micrograph of an epithelial follicles (FE) of *Aedes aegypti* having large nuclei (N). Beneath the epithelial follicles lies the oocyte (OOC) having irregular shape of nucleus (N). **X 2 100**. **B.** Mitochondria (M) as well as several unoriented microvilli (MV) are present in the epithelial follicles. **X 14 250**. **C.** Desmosome (D**)** which joins the epithelial follicles. **X 42 000**.

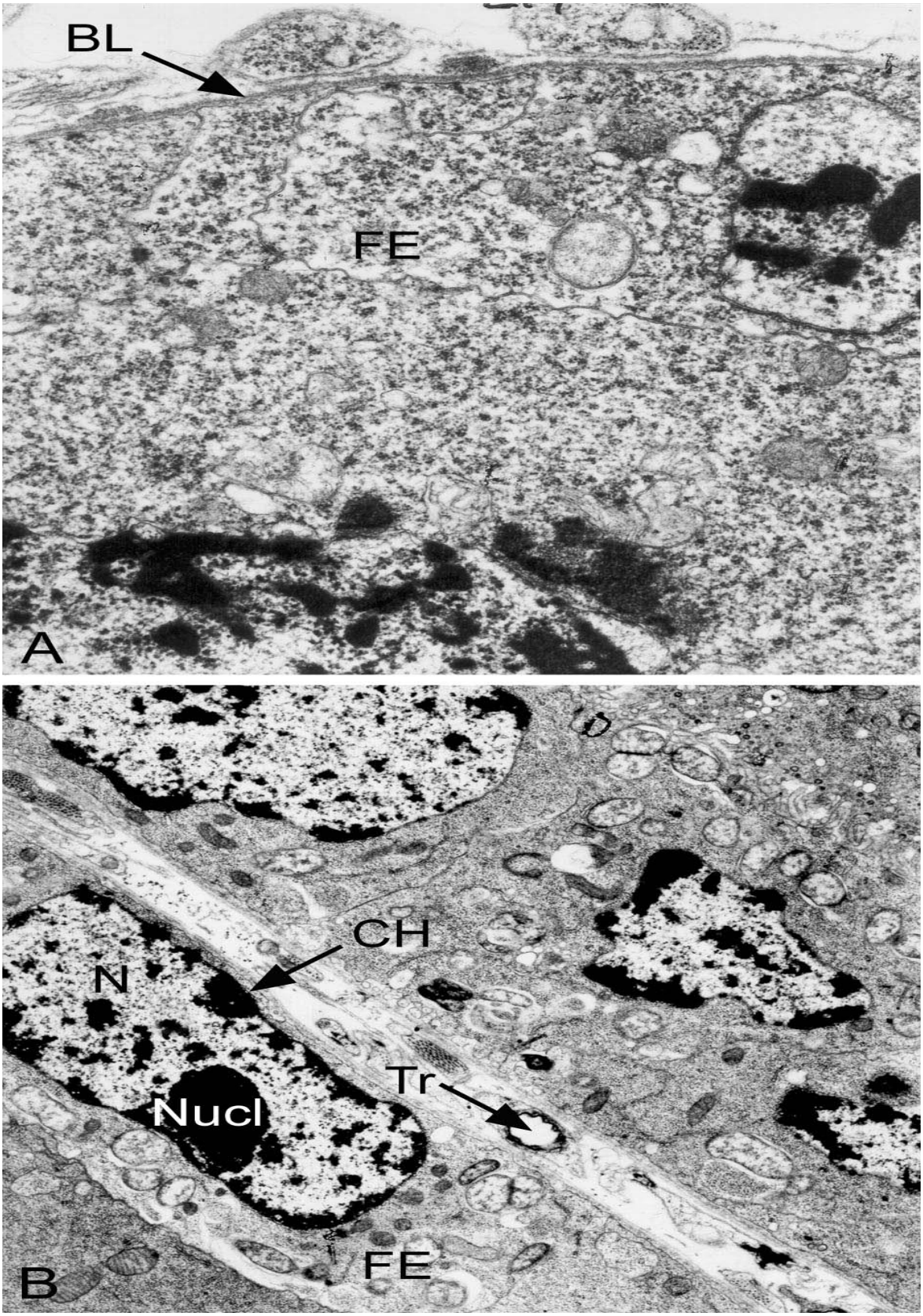


Fig. 3-2A. Transmission electron micrograph of a basement membrane or basal lamina (BL) in which the basal surface of follicle epithelial cells (FE) are lying in the ovary of *Aedes aegypti*. **X 14 250**. **B.** An epithelial follicles of *Aedes aegypti* with nucleus (N) that is enveloped by chromosomal mass (CH) and posseses a distinct polymorphic nucleolus (Nucl). Tracheoles (Tr) are present outside the basament membrane of epithelial follicle (FE). **X 7 125**.

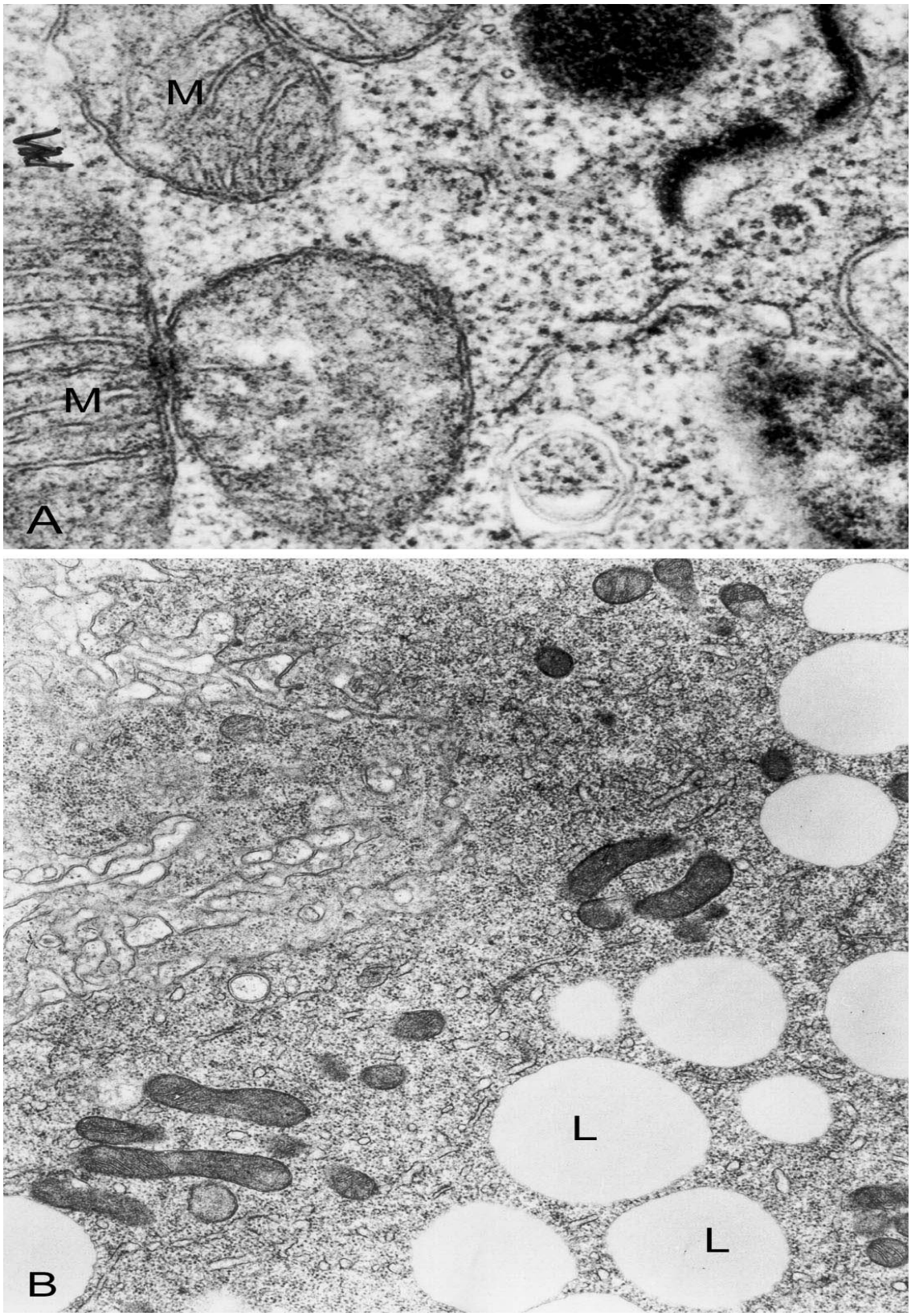


Fig. 3-3A. Transmission electron micrograph of a mitochondria (M) in an ovarian follicle of *Aedes aegypti*. **X 42 000**. **B.** Lysosomes (L) in an ovarian follicle of *Aedes aegypti*. **X 14 250**.

Beneath the epithelial follicles, a single oocyte and a nurse cell are observed (Fig. 3-4A) The nurse cell are relatively large (27μ) with the nuclei alone having the width of three follicular

epithelial cells (Fig. 3-4A & B). The granular nucleoplasm has several discrete nucleoli which is limited by a nuclear envelope and by a large number of pores (Fig. 3-4A).

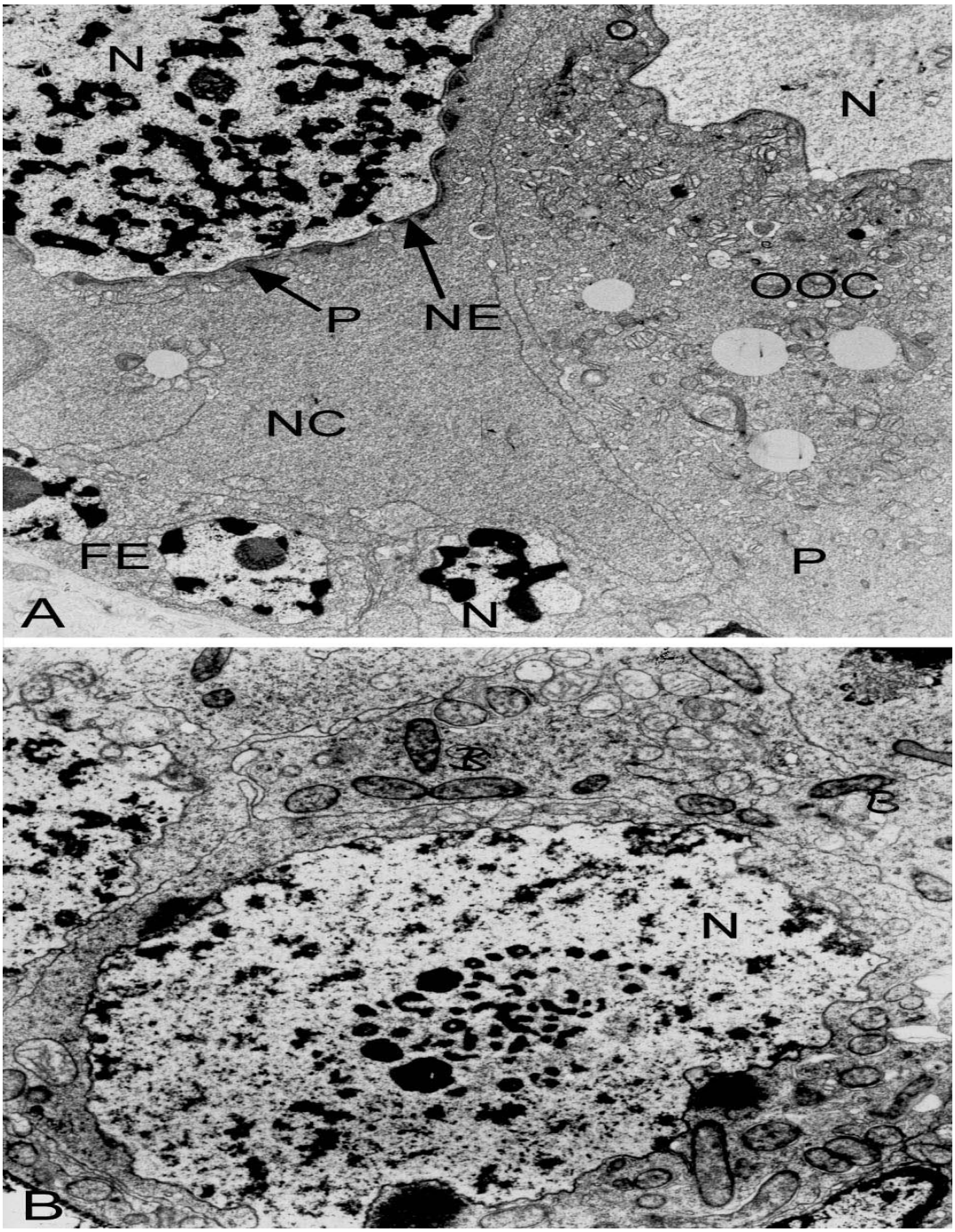


Fig. 3-4A. Transmission electron micrograph of an oocyte (OOC) and nurse cell (NC) which reside beneath the epithelial follicles (FE) in the ovary of *Aedes aegypti*. **X 3 600**. Nurse cell (NC) showing the large nucleus (N) with nuclear envelope (NE) and nuclear pores (P) in the ovary *Aedes aegypti*. **X 7 125**. **B.** Nucleus (N) of a nurse cell in the ovary of *Aedes aegypti*. **X 7 125**.

The oocyte is identified by its irregularly shaped nucleus (Fig. 3-5A). The nucleolus is a dense, meandering, polymorphic structure which extends long branches out into the nucleoplasm. The chromosomal mass possesses a unique configuration of synaptic chromosomes (Fig. 3-5B). The enveloping nuclear membrane is perforated with pores which provides a distinct separation from the rest of the oocytes (Fig. 3-5B).

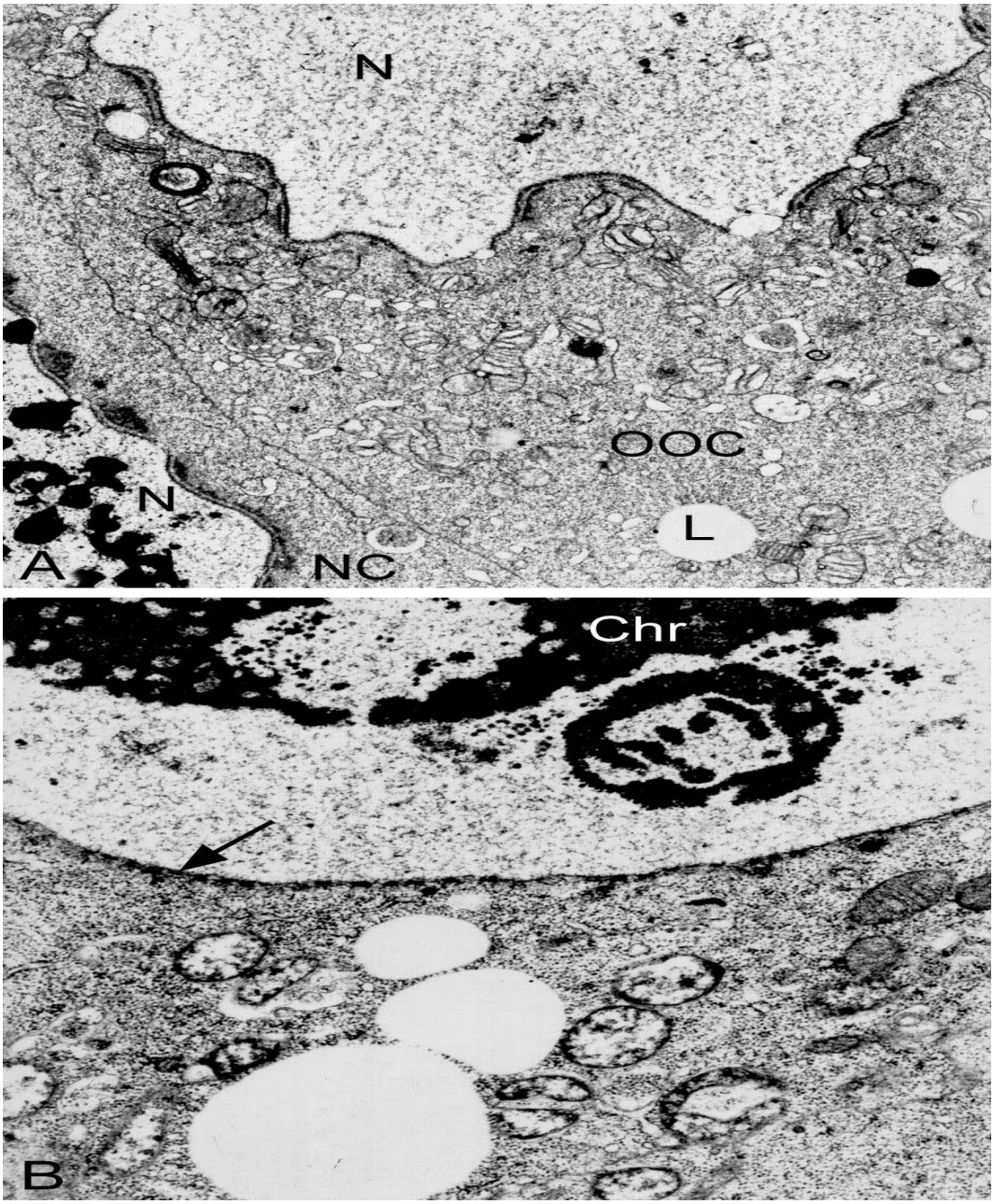


Fig. 3-5A. Transmission electron micrograph of an oocyte (OOC) with irregular nucleus (N) in the ovary of *Aedes aegypti*. **X 14 250**. **B.** Chromosomal mass (Chr) in the nucleus of an oocyte in the ovary of *Aedes aegypti*. **X 42 000**. Nuclear membrane (arrow) in the oocyte of *Aedes aegypti*. **X 42 000**.

There are several pits or vesicular bodies that are present within the border of microvilli. The vesicular bodies are uniformly small and undeveloped as compared with those observed later when yolk deposition begins after blood meal (Fig. 3-6A). Several lysosomes are also present near to the microvilli (Fig. 3-6B).

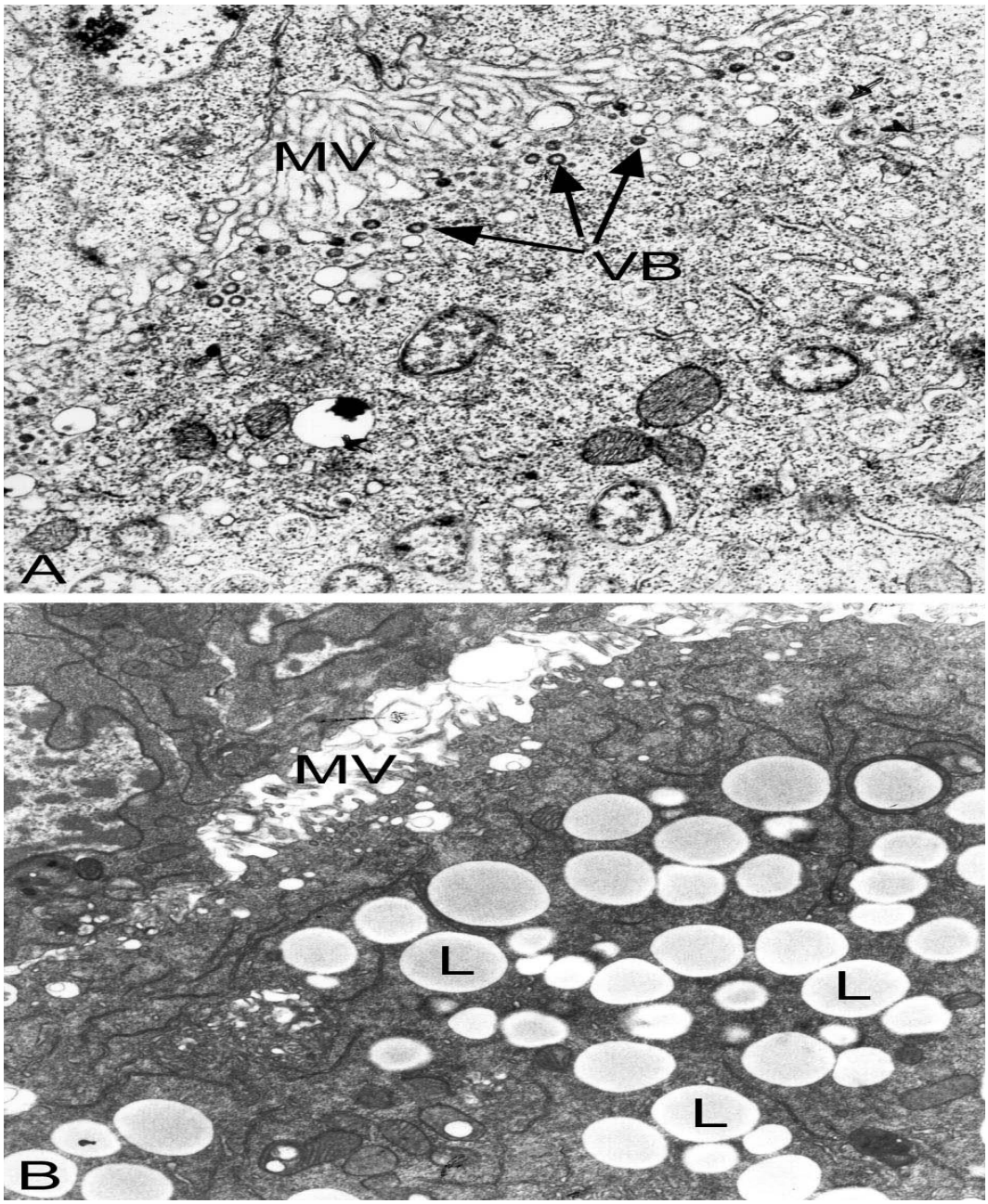


Fig. 3-6A. Transmission electron micrograph of an oocyte having unoriented microvilli (MV) and vesicular bodies (VB) of *Aedes aegypti*. **X 14 250**. **B.** Lysosomes (L) are present near to the microvilli in the oocyte of *Aedes aegypti*. **X 7125**.

The ultra-structure of an epithelial follicle having no blood meal constitutes the base-line on which the substantial changes that occur in the oocyte of the blood-fed mosquito. A meal of blood in the mosquito can alter a series of changes in the cell structure that leads to the formation of a mature egg.

The ultra-structure of mosquito ovaries with blood meal

After the mosquito has taken a blood meal, some prominent changes occur in the epithelial follicles. The very prominent changes that occur are the development of large inter-cellular spaces and a decrease in desmosomal connections which perhaps are associated in the separation of these follicles. The inter-cellular spaces are filled up with finely particulate and somewhat flocculent in appearance (Fig. 3-7A). The distribution of these material throughout the inter-cellular spaces is essentially uniform.

Several numbers of microvilli (also evident during the resting stage but undeveloped) covered the area of the oocyte facing the epithelial follicles of which during seven h after blood meal, push towards the extra-cellular spaces created by the separation of the epithelial follicle cells (Fig. 3-7B). The microvilli vary in length and are not regularly distributed like those of the typical intestinal epithelium. It seems, however, that they have a uniform diameter (60 μm) especially in their greatest extension from the surface (Fig. 3-7B).

Several small protein bodies are observed after the mosquito has taken a blood meal (Fig. 3-8A). A unit membrane is seen which later fuses to form a yolk (Fig. 3-8B). Through the fusion of various units the larger protein bodies of the mature oocyte developed (Fig. 3-8C).

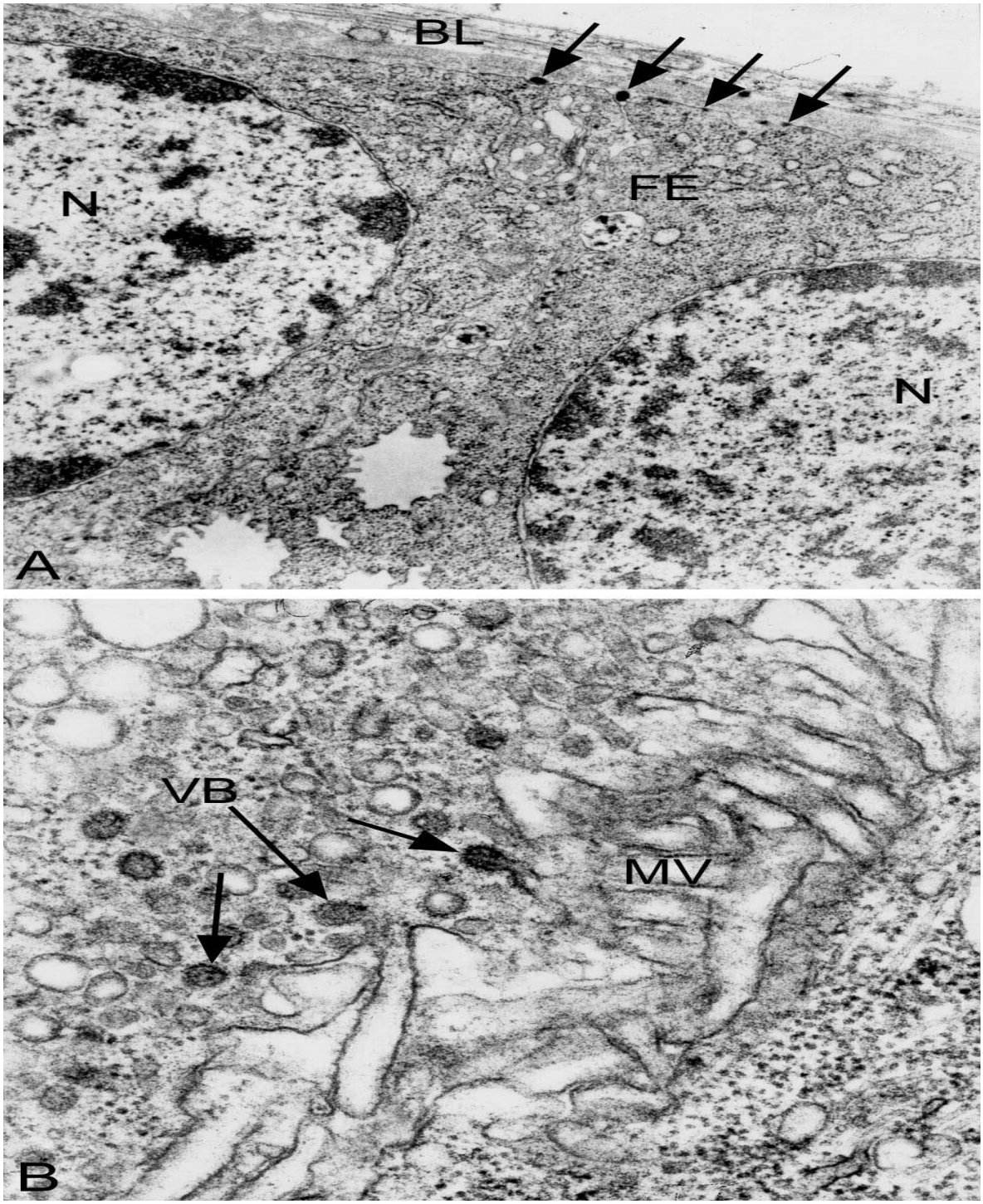


Fig. 3-7A. Transmission electron micrograph of inter-cellular spaces (arrows) in the epithelial follicles (FE) of *Aedes aegypti*. **X 14 250**. **B.** Microvilli (MV) and the vesicular bodies (VB) in the ovary of *Aedes aegypti*. **X 14 250**.

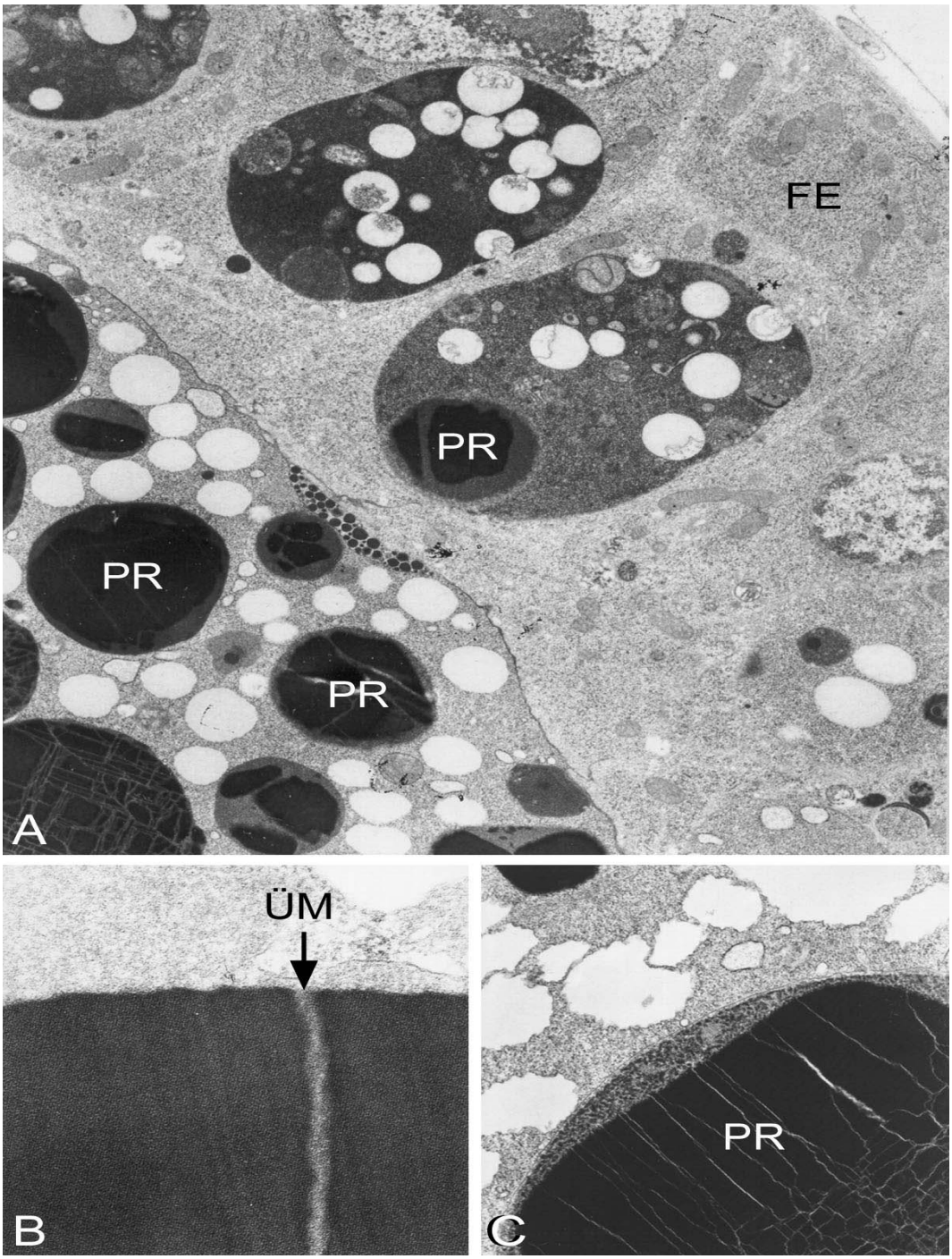


Fig. 3-8A. Transmission electron micrograph of protein bodies (PR) in a mature oocyte of *Aedes aegypti*. **X 42 000**. **B.** Unit membrane which later fuses to form a yolk (UM). **42 000**. **C.** Yolk granules (PR) in the ovary of *Aedes aegypti.* **X 14 250**.

3.3.2. Scanning electron microscopy

The following scanning electron micrographs presented below were taken in order to understand the general structure of the mosquito ovaries.

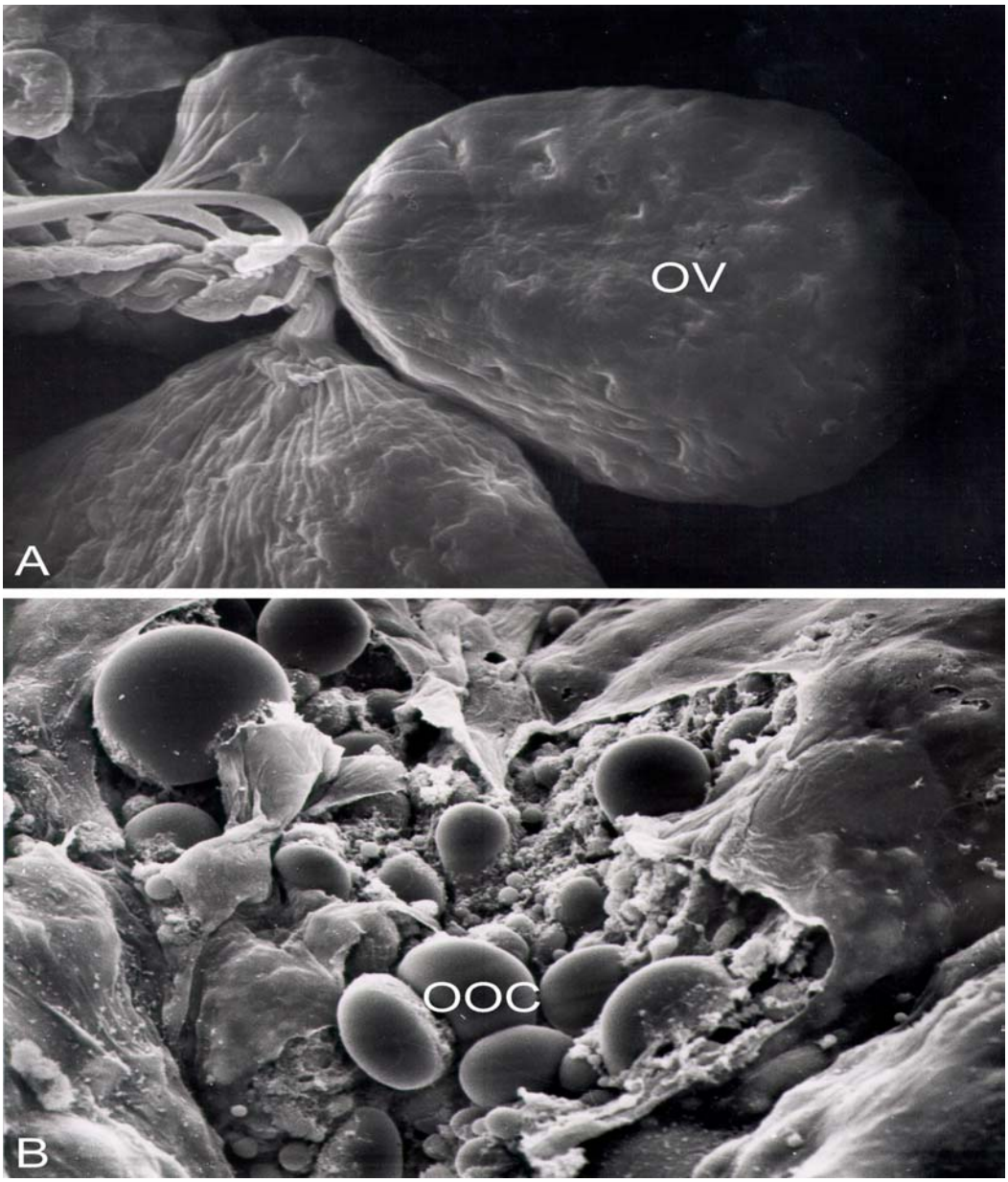


Fig. 3-9A. Scanning electron micrograph of the ovarioles (OV) of female *Ochlerotatus rusticus*. **X 1 550**. **B.** Oocytes (OOC) of female *Ochlerotatus rusticus*. **X 1 050**.

3.3.3. Light microscopy

Two clusters of ovarioles are located in the ovary. These clusters of ovarioles are situated centrally and are surrounded with spongy fat body which encircles the abdomen beneath the cuticle (Fig. 3-10A).

A presumptive follicle is joined to each maturing follicle in the region of nurse cells (Fig. 3-10B). Within the ovariole, a clear, non- staining lipid inclusion in its cytoplasm is observed. This is the oocyte in which the nucleus is surrounded by a refractile lipid droplets (Fig. 3-11A). Nurse cell is also present besides the oocyte. At the periphery of the oocyte, a light region is prominent which is just within the follicular epithelium. This region is the zone of yolk protein uptake (Fig. 3-11B).

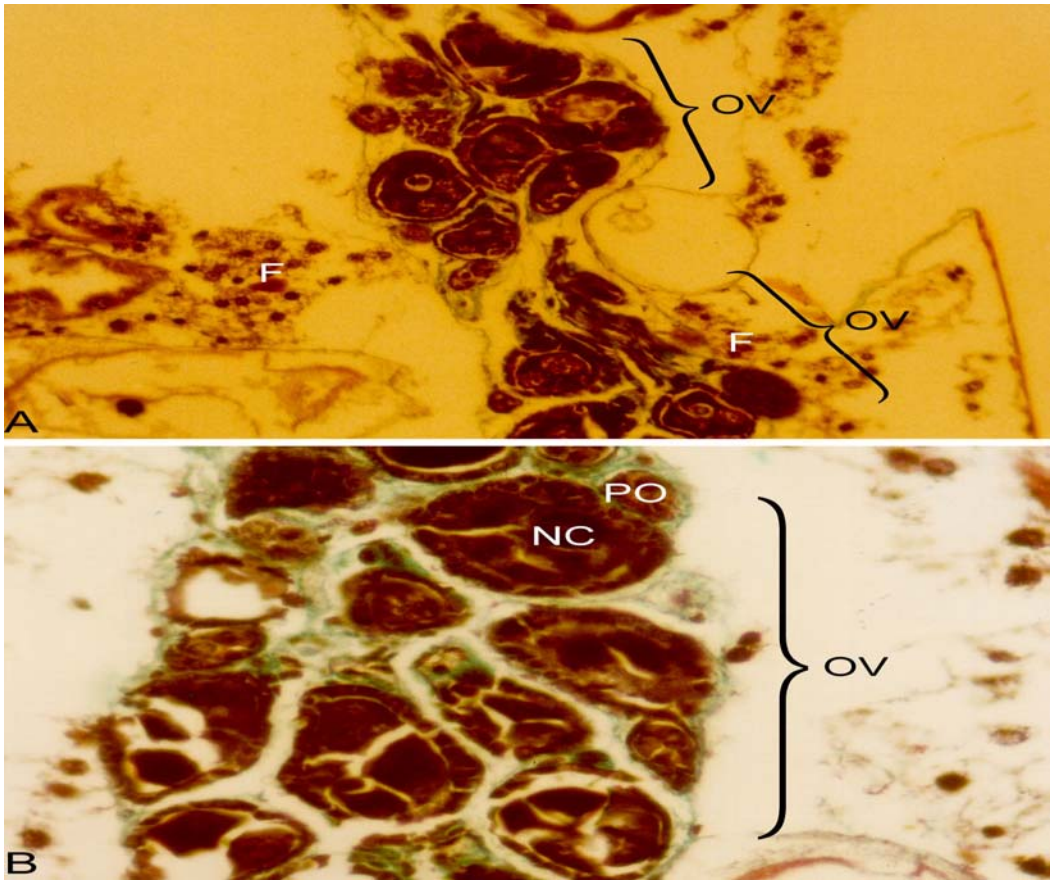


Fig. 3-10A. Light micrograph of the ovary of *Aedes aegypti* showing two clusters of ovarioles (OV) with spongy fat body (F) which surrounds the entire clusters of ovarioles of *Aedes aegypti.* **X 2 500 B.** An ovariole (OV) showing the presumptive follicle (PO) which is attached to the nurse cell (NC). **X 5 000**. Both after Goldner stain.

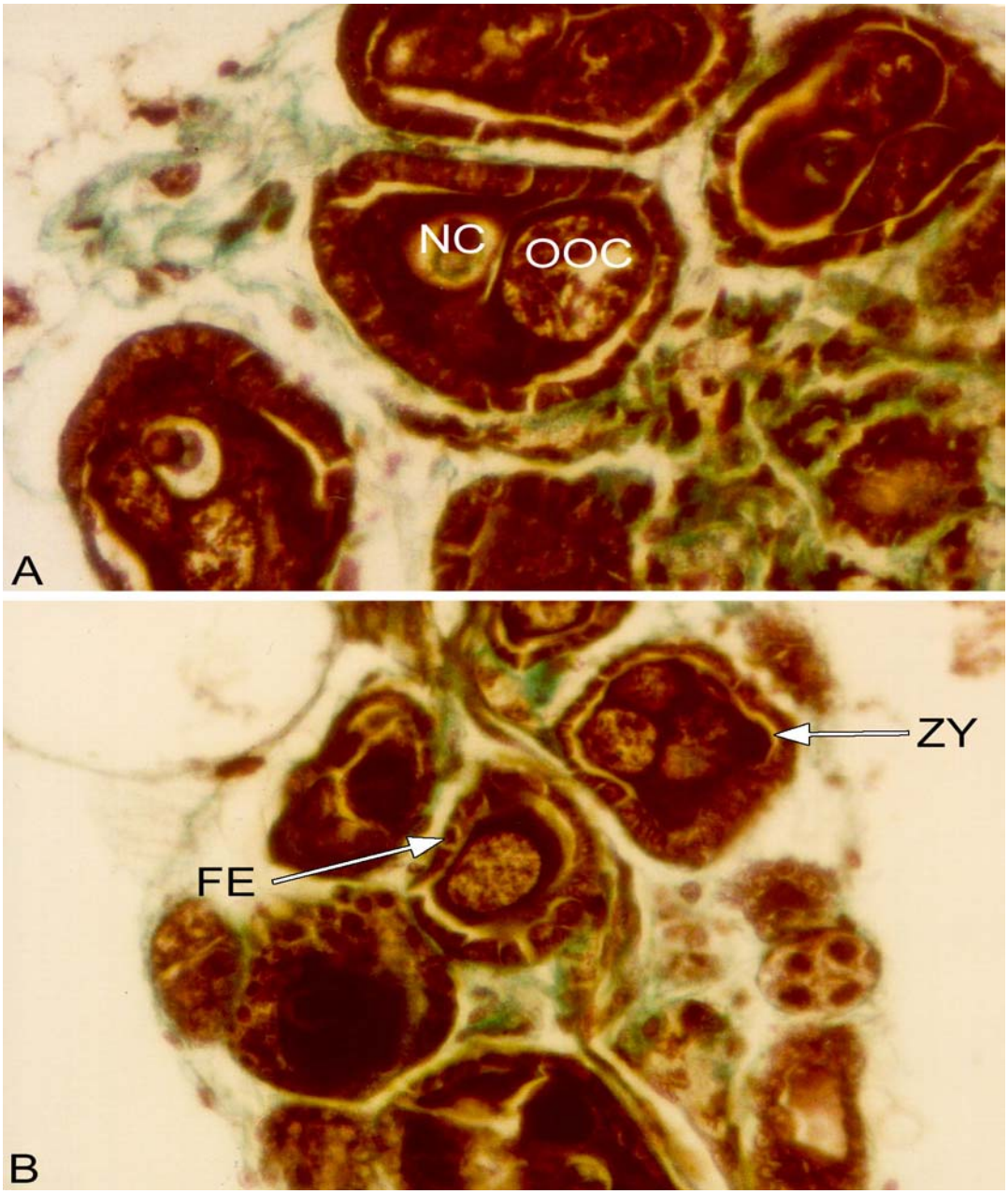


Fig. 3-11A. Light micrograph of an oocyte (OOC) and nurse cell (NC) of *Aedes aegypti*. **X 10 000**. **B**. Zone of yolk protein uptake (ZY) in the epithelial follicle (FE) in the oocyte of *Aedes aegypti*. **X 7 5000**. Both after Goldner stain.

3.4. DISCUSSION

A meal of blood or controlled diet leads to a series of changes in cell structures in the reproductive organ of female mosquitoes that quickly results to the formation of matured eggs. One of the prominent changes observed in the ovary of mosquitoes having blood meal is the presence of various unoriented microvilli in the area of the oocyte which is adjacent to the epithelial follicles (Fig. 3-7B). These microvilli can also be observed in ovary of mosquitoes

having no blood meal but they are smaller in size. Roth and Porter (1964) observed the same structural changes and they cited that the great numbers of microvilli are push into the extra-cellular spaces after 7 h of a blood meal. As it is depicted in this study, these microvilli are somewhat not uniform in lenght and are not regularly dispose as in typical intestinal epithelium which was described by Brandt (1958).

Other prominent structural changes are observed in the region of the oocyte and epithelial follicles of the ovary. These changes are the occurrence of large intercellular spaces and the decrease in desmosomal connections in the epithelial follicle cells (Figs. 3-7A & 3-1B,C). According to Roth & Porter (1964), the epithelial follicle cells open channels between the extra-epithelial follicle space and the surface of the oocyte for the exchange of materials for the development of yolk

Furthermore, pits or vesiclular bodies are developed after a mosquito has taken a blood meal (Fig. 3-6A). Pits of similar structure obtained from this study have been reported in many cell types (Anderson, 1962; Roth & Porter, 1962; Roth & Porter, 1963). They are not to be confused with the simpler pits found especially in smooth muscle cells and blood vascular endothelial cells. The latter have simple, clean limiting membranes and are smaller in diameter (Palade & Siekevitz, 1956).

The observations of pits in this study are in agreement with some other authors (Clements, 1992; Roth & Porter, 1964). These authors interpreted that these pits are engaged in pinocytocis which is associated in the uptake of materials for yolk formation. In the study done by Roth and Porter (1964), it was found that the number of pits approximately 300,000 are observed in the oocyte after 7 h of a blood meal. This results in a 15 times increase of the number of pits found in the oocyte after the mosquito has taken a blood meal. Pits or vesicular bodies fuse to form small crystalline yolk droplets which subsequently coalesce to form large proteid bodies (Roth & Porter, 1964). Apparently, through the fusion of these various units of proteid bodies, the mature oocyte developed as depicted in this study in Figure 3-8A, B and C. The implication from the above observations and the literature references (Chargaff, 1942; Brambell & Hemmings, 1954; Smith, 1959; Christophers, 1961; Caro & van Tubergen, 1962) on yolk synthesis is that these pits are responsible for taking up materials from the extracellular space of the follicle and contribute it to yolk granule formation.

Likewise, the results of scanning electron microscopy confirm the structure of the ovaries described by Clements (1992). The number of ovarioles ranges from 50 to 500 (Fig. 3-9A & B) depending on the species, the physiological stages as well as the size of the individual female (Clements, 1992). Clusters of ovarioles are surrounded with a spongy fat body as depicted in this study by using light microscopy (Fig. 3-11A). This observation is in agreement with Roth and Porter (1964) who described the yolk protein uptake in the oocyte of the mosquito *Aedes aegypti*.

There are reasons to believe that yolk deposition in the developing oocyte of a mosquito is accomplished by the removal of the protein from the blood (Smith, 1968). All of the structural mechanisms associated with rapid synthesis of proteins or lipoproteins, especially for segregation and storage in granules, appear in the fine structure of the epithelial follicles. Indeed, the very unsusual structural feature does appear, which is seemingly involved in the yolk deposition, are the development of pits or vesicular bodies (Clements, 1992). Generally, yolk develops rapidly, in fact, synthesis and storage are essentially completed at least 25 h after blood meal (Roth & Porter, 1964; Singh & Brown, 1957). By 4 h after the blood meal, these changes in the cell structure are already in evidence, and after 7 h they are very obvious (Roth & Porter, 1964).

The present light, transmission and scanning microscopy play a valuable part in revealing some events of oogenesis. Futhermore, these instruments give various insights concerning to the ultrastructures of the ovaries which serve as a basis for the identification of *Wolbachia sp*. infection (Chapter 4).

4. Molecular and electron microscopic identification of *Wolbachia* in Culex Pipiens Complex populations from the Upper Rhine Valley, Germany, and Cebu City, Philippines

4.1. INTRODUCTION

4.1.1. General background of *Wolbachia*

Human diseases such as typhus, Rocky Mountain spotted fever, and Q-fever are caused by Rickettsiae which are obligate intracellular microorganisms that are associated with arthropod vectors (Tai Min & Benzer, 1997). Various insects are known to have intracellular infection with rickettsia-like microorganisms of the genus *Wolbachia* (Hoshizaki & Shimada,1995). *Wolbachia* is a bacterium which belongs to the tribe Wolbachiae and family Rickettsiaceae of the order Rickettsiales. They are a common and widespread group of bacteria which are found in reproductive tissues of arthropods (Werren, 1997). *Wolbachia* is known to alter the reproduction of its host and have attracted much attention by virtue of its ability to manipulate the reproduction of its arthropod hosts (Hurst, et al., 2000; Jiggins et al., 2001).

There are about 15-20% of all known insect genera that are estimated to contain *Wolbachia* which are maternally inherited (Tai-Min & Benzer, 1997; Rasgon & Scott, 1999). They are obligate intracellular symbionts which appear to be universally present in many invertebrate species including mosquito namely: *Culex, Aedes* and *Armigeres* (Clements, 1992; Jammongluk, 2000).

Infections of *Wolbachia* were first observed in the ovaries and testes of *Cx. pipiens* by using light microscopy. It was named and described in the same insect by Hertig (1936). *Wolbachia* species are not only restricted to insect hosts, however, they are also observed in certain groups of invertebrates such as millipedes, crustaceans, and mites (Zimmer, 2001). Furthermore, they are also observed in the phylum Nematoda (Stouthamer et al., 1999). These *Wolbachia* species form a separate sister clade to their arthropod counterparts and appear to be mutualist rather than reproductive parasite (Sironi et al., 1995; Bandi et al., 1998; Taylor & Hoerauf, 1999; Hoerauf et al., 2000).

Wolbachia species in insects have been long implicated in the phenomenon of cytoplasmic incompatibility (CI), in which certain crosses between symbiont-infected individuals lead to embryonic death or sex ratio distortion (O'Neill et al., 1992). This phenomenon has been observed in different insect species including *Culex pipiens* which was proven to be infected with *Wolbachia pipientis* (Yen and Barr, 1971;1974). However, *Wolbachia* species do not always induce CI in their hosts but also cause feminization in isopods and parthenogenesis in wasps (Werren 1997; Bouchon et al., 1998; Stouthamer et al.,1999).

Wolbachia pipientis in mosquitoes is transmitted through the cytoplasm of the eggs from one generation to the next (Yen and Barr, 1971; Yen, 1975; Wright and Barr, 1981; Werren, 1997). In the ovaries of mosquitoes, infections of *W. pipientis* occur in both oocytes and nurse cells in forms of rods and cocci (rounded cells). The coccoid forms are up to 1.1 μm or more in diameter and the rods are variable in forms and are approximately 0.3-0.7 μm in diameter and 1.1-2.2 μm in length. *Wolbachia* divides by binary fission within the vacuoles of the host cells (Clements, 1992). They are present in all life stages of mosquitoes from the newly-laid eggs to the adult mosquitoes. In recently-laid eggs, *Wolbachia* is concentrated near the micropyle and as the embryo develops, *Wolbachia* infections become more restricted to the pole cells. During the 3rd and 4th instar larval stages, the bacteria multiply rapidly (Clements, 1992).

The ultrastructure of *Wolbachia* is typical of gram-negative bacteria. It has two cell membranes, surrounded by a third membrane which is thought to be of host origin. The two membranes surrounded each symbiont, namely: a plasma membrane and an outer cell wall. Many ribosomes and strands of DNA are present in the cytoplasm. In Culex Pipiens Complex, each symbiont is surrounded by a membrane of host origin. In species of *Aedes scutellaris*, the host membrane was described as present or absent by different investigators (Yen, 1975; Wright et al., 1978; Beckett et al., 1978; Wright and Barr, 1980).

4.1.2. Effects of *Wolbachia* infections on the fertilization and embryonic development in mosquitoes

Wolbachia species are widely distributed intracellular bacteria that cause a number of reproductive alterations in their eukaryotic hosts (Werren & Bartos, 2001). They can be responsible for the selective sweeps on mitochondrial DNA variablility within species (Rokas

et al., 2001). Although they can live only inside their host's cells, transmission is enhanced which involved a large scale of host manipulation like CI that caused sterility in cross matings (Stouthamer et al., 1999; Curtis & Sinkins, 1998). *Wolbachia's* most common effect is the crossing incompatiblity between infected males and uninfected females. Nothing is known about the interactions of these bacteria with the host's immune system (Bourtzis et al., 2000). In the 1970's through the process known as CI, it has been shown that the presence of *Wolbachia* caused difficulties for uninfected females to reproduce when a healthy female mates with a male carrying *Wolbachia.* Some or all of the fertilized eggs will die. But a female carrying *Wolbachia* can mate with either infected or uninfected males and produce viable eggs all with *Wolbachia* infections (Zimmer, 2001). As a result, CI gives infected females a reproductive advantage and allows *Wolbachia* to spread rapidly through insect populations. Additionally, *Wolbachia* has been suggested to be an important factor in arthropod evolution, from host speciation to the evolution of sex-determination systems (Rasgon & Scott, 1999; Hurst et al., 2000).

4.1.3. Host range and phylogeny of *Wolbachia*

With the use of Polymerase Chain Reaction (PCR) for the detection of *Wolbachia* by amplifying the 16S rDNA, rapid accumulation of data on the host range of *Wolbachia* was possible (O'Neill et al., 1992; Holden et al., 1993; Werren et al., 1995).

Sequence analyses based on 16S rRNA have confirmed that morphological similarities to the Rickettsia are based on phylogenetic relatedness and revealed that within the alpha-Proteobacteria, *Wolbachia* is most closely related to the *Erlichia* clade (O'Neill et al., 1992, Rousset et al., 1992).

With the wide host range of *Wolbachia* species and their clear evidence for inter-specific transfer, *Wolbachia* has shown to be a possible tool for the exploration of potential applications as a gene drive mechanism of broad applicability. This bacteria have also an extraordinary ability to manipulate their host for their own evolutionary benefit that can help to turn a population of hosts into a new species. They could also be of great interest as a potential tool for biological control and genetic manipulation of pests and disease vectors of wild insect populations, and as a new method for interfering diseases caused by filarial nematodes (Stouthamer et al.,1999 ; Werren & Bartos, 2001).

Furthermore, *Wolbachia* can be used as a weapon against parasites that cause disease such as malaria (Zimmer, 2001). To fight malaria, for example, researchers might be able to introduce a gene encoding resistance to *Plasmodium* sp. into *Wolbachia's* genome. Researchers might then infect the mosquitoes with *Wolbachia* which could theoretically produce substances like enzymes that inhibit the transmission of the parasite through the insect's body (O'Neill, 1999). Taking a different strategy to fight malaria, a virulent strain of *Wolbachia* exists that infects *Drosophila melanogaster* cutting its life-span up to 50%. Upon killing insects before human parasites develop, the transmission process is interrupted. Under certain conditions, 80 to 100% reduction of diseases transmission will be observed (O'Neill, 1999).

In this study, PCR and transmission electron microscopy were used to detect the presence of *Wolbachia* in populations of the Culex Pipiens Complex from the Upper Rhine Valley, Germany, and Cebu City, Philippines. Furthermore, individuals of *Drosophila melanogaster* that were proven to be infected with *Wolbachia* were used as model specimens for the electron microscopic study.

4.2. MATERIALS AND METHODS

4.2.1. *Drosophila melanogaster* strain

Five cultured individuals of a laboratory *D. melanogaster* strain (carrying the genetic markers white, black cell, glaze eyes and curly wings) that were infected with *Wolbachia* were used as model for electron microscopic study. The insect's strain was provided by Dr. Dirk Lankenau, Institute of Zoology, University of Heidelberg, Germany.

4.2.2. Mosquito strains

Mosquito individuals from five populations of adult *Cx. pipiens* (L) from the Upper Rhine Valley, Germany, and one population of *Cx. quinquefasciatus* (Say) from Cebu City, Philippines were used in this study. Larvae were collected from various breeding sites and reared to the adult stage in the laboratory. Each population was assigned with a corresponding

accession letter. Individuals referring to accession letters P and KL were collected from Promarkt, Mainzerstrasse and Theodor Heuss Ring, respectively, both from Wiesbaden , State of Hessen, Germany. Individuals referring to accession letters L and SO were collected from Laubertsweg, Altenheim and Schwanau-Ottenheim, respectively, both from State of Baden-Württemberg, Germany. Individuals referring to accession letter B were collected from Bergweg, Mainz-Bretzenheim, State of Rheinland-Pfalz, Germany and individuals referring to accession letter CP were collected from Cebu City, Philippines.

Species referring to populations with accession letters P, L, and KL were collected from underground breeding sites while all other species were from above breeding surface breeding sites (Table 4-1 & Figs. 4-1A & B). All populations that were from the underground breeding sites were proven to be autogenous based on rearing tests while all populations from the above ground surface breeding sites were unautogenous because the females needed a blood meal for reproduction (Becker et al., 1999).

Fig. 4-1A. A cesspool which is a typical underground breeding site**.**

Fig. 4-1B. Water containers which are typical above ground breeding site.

4.2.3. Preparation of mosquito samples

Twenty to sixty adult mosquitoes from each population including both sexes were used in the study. The whole adult males were used for PCR assays while adult females were sectioned into two parts. The heads were used for PCR assays and the abdomens for electron microscopic study when an infection was proven by PCR.

4.2.4. DNA extraction

Whole adult males and abdomen of females were placed in 1.5 ml Eppendorf tubes, homogenized by using a plastic pestle in 100 µl extraction buffer (1M Tris-HCl pH 9.0, 0.5 M EDTA, 20% SDS and 1% DEPC) and incubated at 70^{o}C for 30 min. To each tube, 37.5 µL of 3M potassium acetate were added. Homogenized specimens were placed on ice for 30 min. and centrifuged at 4^{o}C (12,000-14,000 rpm) for 15 min. Supernatants were transferred into new tubes and cellular debris were discarded. Genomic DNA was precipitated by the addition of 200 µl of 96% ethanol. Tubes were again centrifuged at 14,000 rpm for 5 min. to pellet the DNA. Supernatants were carefully discarded by not disturbing the DNA pellets. Pellets were

washed once with 70% EtOH, air dried and resuspended in 100 µl TE at 37^{o}C for 15 min. DNA samples were stored at 4 ^{o}C until further test.

4.2.5. PCR assay

PCR assays were conducted by using two primers. Primer set 1 amplified a 0.9 kb fragment from *Wolbachia* 16S rDNA and is specific to *Wolbachia* (forward: 5'TTGTAGCCTGCTATGGTATAACT-3', reverse:5'GAATAGGTATGATTTTCATGT-3'). Primer set 2 amplified a 0.4 kb fragment from insect mitochondrial 12S rDNA (forward:5' CTAGGATTAGATACCCTATT-3', reverse: 5'AAGAGCGACGGGCGATG-3'). The second primer set is universal for insect mitochondrial DNA and was used as a control to check the quality of each DNA extraction. The samples were amplified in an Eppendorf PCR Master Cycler machine by using the following protocol: 1 cycle of 95^{o}C for 5 min. followed by 30 cycles each of: 95^{o}C for 1 min., 54^{o}C for 1 min., 72^{o}C for 1 min. After amplification, samples were held at 72 ^{o}C for 5 min. and stored at 4 ^{o}C until usage.

PCR products were separated with 1% agarose gel by electrophoresis, stained with ethidium bromide and visualized under UV light. Gels were photographed for permanent record of results.

4.2.6. Transmission electron microscopy (TEM)

A. Preparation of mosquito samples

Abdomens of infected adult female mosquitoes were fixed by using 2.5 % glutaraldehyde in 0.1 cacodylate buffer (pH 7.4) for a period of 2 h. Fixation was continued for a period of 3 to 4 h at 4^{o}C. Tissues were dehydrated in an increasing concentrations of alcohol (50, 70, 80, 96 and 100%). After dehydration, the tissues were infiltrated overnight in a 1:1 mixture of Spurr pur and absolute alcohol which was followed by 3:1 mixture of Spurr pur and absolute alcohol. Tissues were embedded in Spurr pur and polymerized at 60^{o}C for 24 h.

Ultrathin sections (60-90 µm) were cut by using Cambridge Huxley microtome and examined under Zeiss electron microscope.

B. Preparation of *D. melanogaster*

The abdomens of *D. melanogaster* that were infected with *Wolbachia* were fixed in 2.5% glutaraldehyde in 0.1 cacodylate buffer (pH 7.4). Oocytes were removed from the body and were used for electron microscopic study. Fixation was done by using the same procedures applied in mosquito samples.

4.3. RESULTS

4.3.1. PCR assays of *Cx. pipiens* and *Cx. quinquefasciatus*

PCR assays showed that all *Cx. pipiens* populations from the Upper Rhine Valley, Germany as well as *Cx. quinquefasciatus* from Cebu City, Philippines were infected with *Wolbachia pipientis*. Results of the study are summarized in Table 4-1. Individuals that were positive for *Wolbachia* infections exhibited bands referring to the *Wolbachia*-specific fragment at 0.9 kb, and the insect mitochondrial DNA control fragment at 0.4 kb. For *Wolbachia*-negative individuals, only the control band was detected.

Results showed that species from all populations of Culex Pipiens Complex were infected with *Wolbachia* at different rates. Highest infection rate of 100% was observed in females of the population corresponding to accession letter P. This was followed by an infection rate of 70% in females belonging to accession letter KL. Other females showed infection rates of 60%, 20% and 10% in the populations corresponding to accession letters L, SO, and B, respectively.

Highest infection rate of 50% was observed in males from population with accession letter P. This was followed by males from population with accession letter KL infected at a rate of 30%. Males of both populations with accession letters L and B showed the same infection rate of 20% while the lowest infection rate of 10% was detected in the population with accession letter SO (Table 4-2 & Fig. 4-1A to 4-6A, B, respectively).

Males of *Cx. quinquefasciatus* from Cebu City, Philippines were infected at a rate of 23.33% while an infection rate of 10 % was observed in females.

Table 4-1. Percentages of *Wolbachia* infections in Populations of the Cx. Pipiens Complex from the Upper Rhine Valley, Germany and Cebu City, Philippines.

Location	Type of breeding site	no. of		no. of infected		% infected	
		male	female	male	female	male	female
P	underground	10	10	5	10	50%	100%
L	underground	10	10	2	6	20%	60%
KL	underground	10	10	3	7	30%	70%
SO	above surface	10	10	1	2	10%	20%
B	above surface	5	10	1	1	20%	10%
CP	above surface	30	30	7	3	23.33%	10%

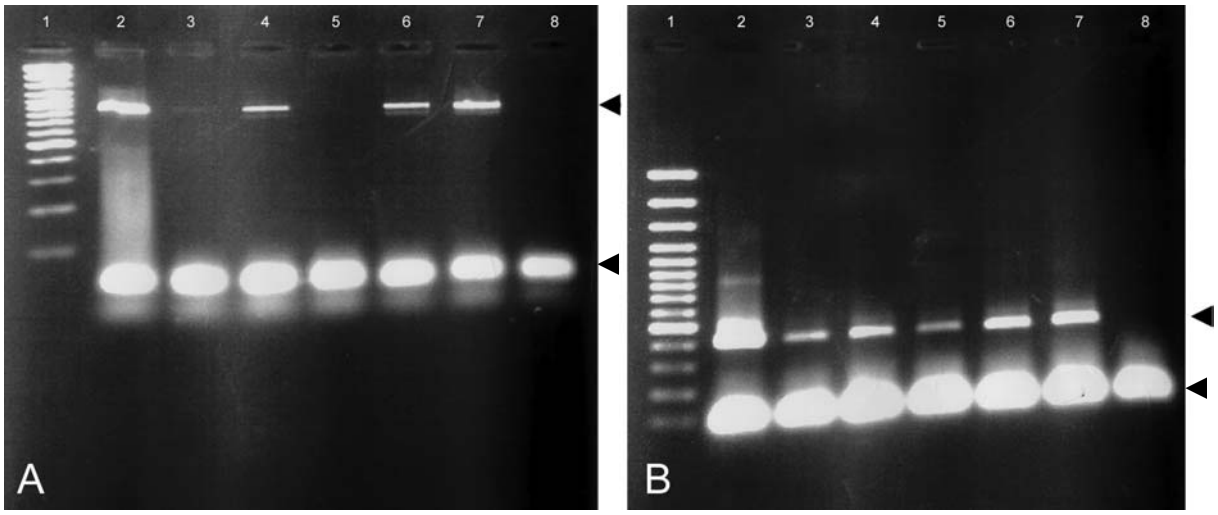


Fig. 4-2A. Example of PCR products of *Cx. pipiens* referring to accession letter P. *Wolbachia*-specific bands are visible at 0.9 kb (upper arrow). Lanes: 1, DNA size markers; 2, *Drosophila*-positive control; 3, *Wolbachia*-uninfected mosquito; 4, *Wolbachia*-infected mosquito; 5, *Wolbachia*-uninfected mosquito; 6, *Wolbachia*-infected mosquito; 7, *Wolbachia*-infected mosquito, 8, UV water negative control. **B**. Insect mitochondrial bands are visible with at 0.4 kb (upper arrow). Lanes: 1, DNA size markers; 2, *Drosophila*; 3 to 7, *Cx. pipiens*; 8, UV water negative control. Lower arrows are unused oligo-nucleotides.

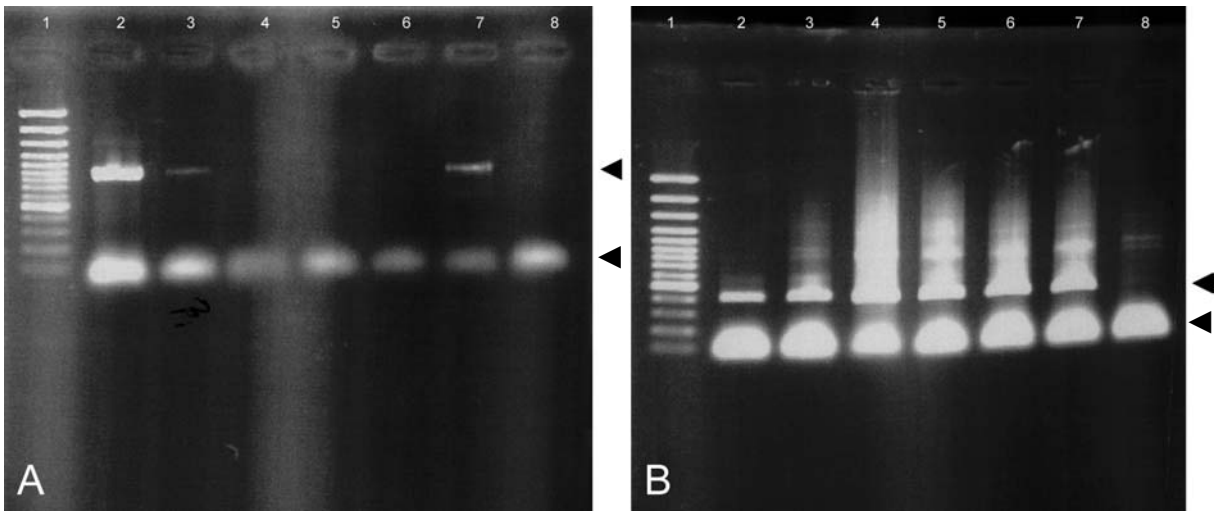


Fig. 4-3A. PCR products of *Cx. pipiens* referring to accession letter B. *Wolbachia* specific bands are visible at 0.9 kb (upper arrow). Lanes: 1, DNA size markers; 2, *Drosophila*-positive control; 3, *Wolbachia*-infected mosquito; 4, *Wolbachia*-uninfected mosquito; 5, *Wolbachia*-uninfected mosquito; 6, *Wolbachia*-uninfected mosquito; 7, *Wolbachia*-infected mosquito; 8, UV water nagative control. **B**. Insect mitochondrial bands are visible at 0.4kb (upper arrow). Lanes: 1, DNA size markers; 2, *Drosophila*; 3 to 7, *Cx. pipiens*; 8, UV water negative control. Lower arrows are unused oligo-nucleotides.

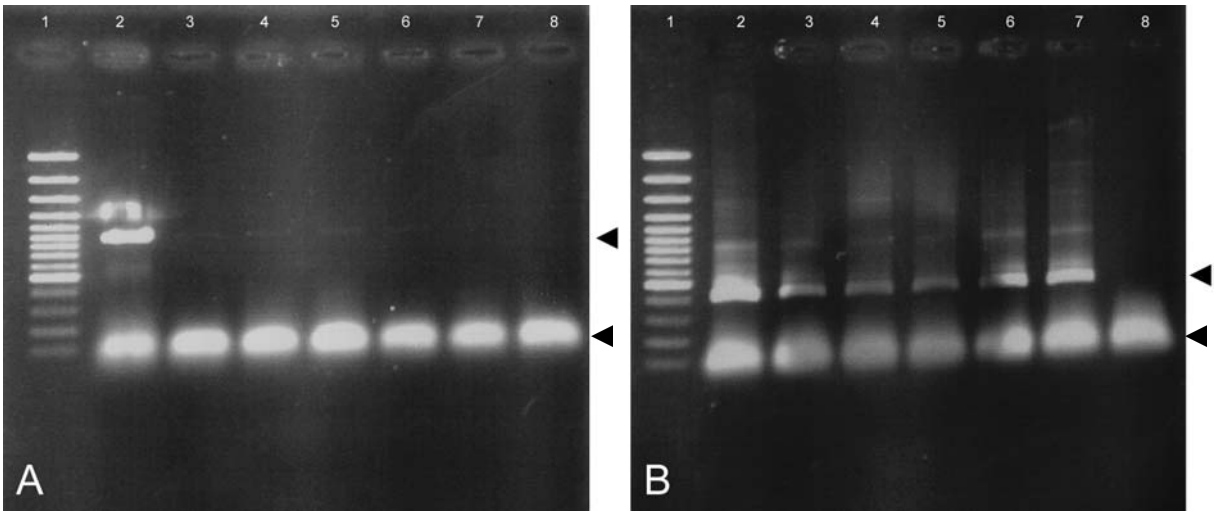


Fig. 4-4A. PCR products of *Cx. pipiens* referring to accession letter L. *Wolbachia* specific bands are visible at 0.9 kb (upper arrow). Lanes: 1, DNA size markers; 2, *Drosophila*-positive control; 3 to 7, *Wolbachia*-uninfected mosquitoes; 8, UV water negative control. **B**. Insect mitochondrial bands are visible at 0.4 kb (upper arrow). Lanes: 1, DNA size markers; 2, *Drosophila*; 3 to 7, *Cx. pipiens*; 8, UV water negative control. Lower arrows are unused oligo-nucleotides.

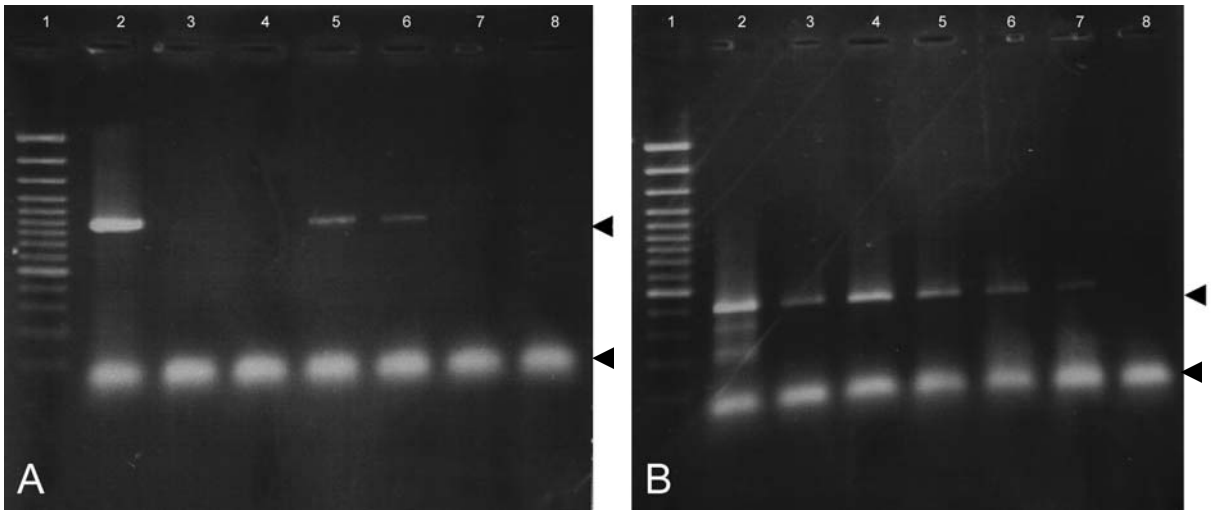


Fig. 4-5A. PCR products of *Cx. pipiens* referring to accession letter SO. *Wolbachia* specific bands are visible at 0.9 kb (upper arrow). Lanes: 1, DNA size markers; 2, *Drosophila*-positive control; 3 & 4, *Wolbachia*-uninfected mosquito; 5 & 6, *Wolbachia*-infected mosquito; 7, *Wolbachia*-uninfected mosquito; 8, UV water nagative control. **B**. Insect mitochondrial bands are visible at 0.4 kb (upper arrow). Lanes: 1, DNA size markers; 2, *Drosophila*; 3 to 7 *Cx. pipiens*; 8, UV water negative control. Lower arrows are unused oligo-nucleotides.

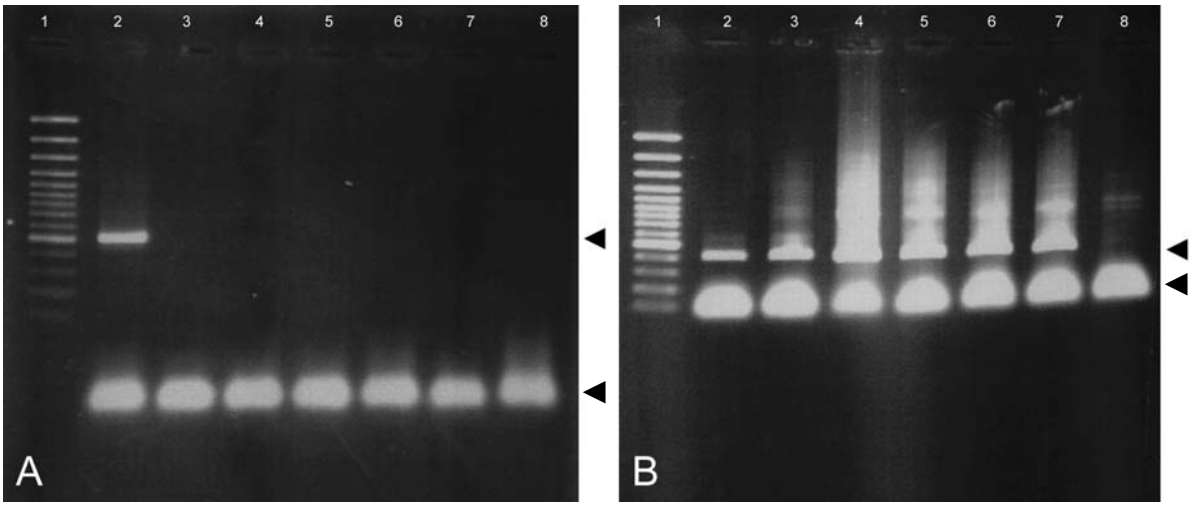


Fig. 4-6A. PCR products of *Cx pipiens* referring to accession letter B. *Wolbachia* specific bands are visible at 0.9 kb (upper arrow). Lanes: 1, DNA size markers; 2, *Drosophila*-positive control; 3 to 7, *Wolbachia*-uninfected mosquitoes; 8, UV water negative control. Fig. 5B. Insect mitochondrial bands are visible at 0.4 kb (upper arrow). Lanes: 1, DNA size markers; 2, *Drosophila*; 3 to 7*, Cx. pipiens*; 8, UV water negative control. **B**. Insect control bands. Lanes: 1, DNA size markers; 2, *Drosophila;* 3 to 7, *Cx. pipiens*; 8, UV water negative control. Lower arrows are unused oligo-nucleotides.

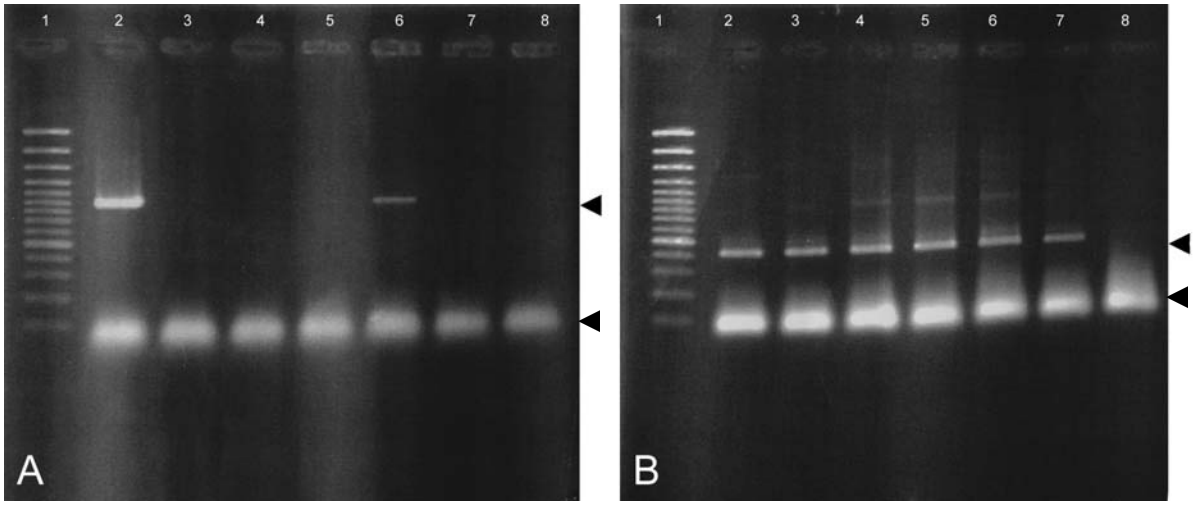


Fig. 4-7A. PCR products of *Cx. quinquefasciatus* referring to accession letter CP. *Wolbachia* specific bands are visible at 0.9 kb (upper arrow). Lanes: 1, DNA size markers; 2, *Drosophila*-positive control; 3 to 6, *Wolbachia*-uninfected mosquitoes; 7, *Wolbachia*-uninfected mosquito; 8, UV water negative control. **B**. Insect mitochondrial bands are visible at 0.4 kb (upper arrow). Lanes: 1, DNA size markers; 2, *Drosophila*; 3 to 7, *Cx. quinquefasciatus*; 8, UV water negative control. Lower arrows are unused oligo-nucleotides.

Transmission electron microscopic studies of the ovary of infected species of the Cx. Pipiens Complex confirmed the presence of *Wolbachia* (Figs. 4-8 to 4-10). The bacteria occurred frequently in the oocytes (Fig. 4-8A). Two different forms of *Wolbachia* cells were observed, namely; rods and cocci (Fig. 4-8B). The bacteria exhibited the typical morphology for *Wolbachia* with three cell membranes comprising the plasma membrane, cell wall and an outermost membrane which is thought to be of host origin (Fig. 4-8C). Within the cytoplasm of the bacteria, various ribosomes and fine strands of DNA were present (Fig. 4-8D) .

4.3.2.Transmission electron microscopy

The ultrastructure of *Wolbachia* in *D. melanogaster* exhibited the same morphology observed in Culex Pipiens Complex. The bacteria are present in the oocytes (Figs. 4-8 A and 4-B). The bacteria exhibited a typical morphology for *Wolbachia* with three cell membranes which has been similarly observed in the mosquitoes (Fig. 4-8C). Within the cytoplasm of the bacteria, various ribosomes and fine strands of DNA were present (Fig. 4-8D).

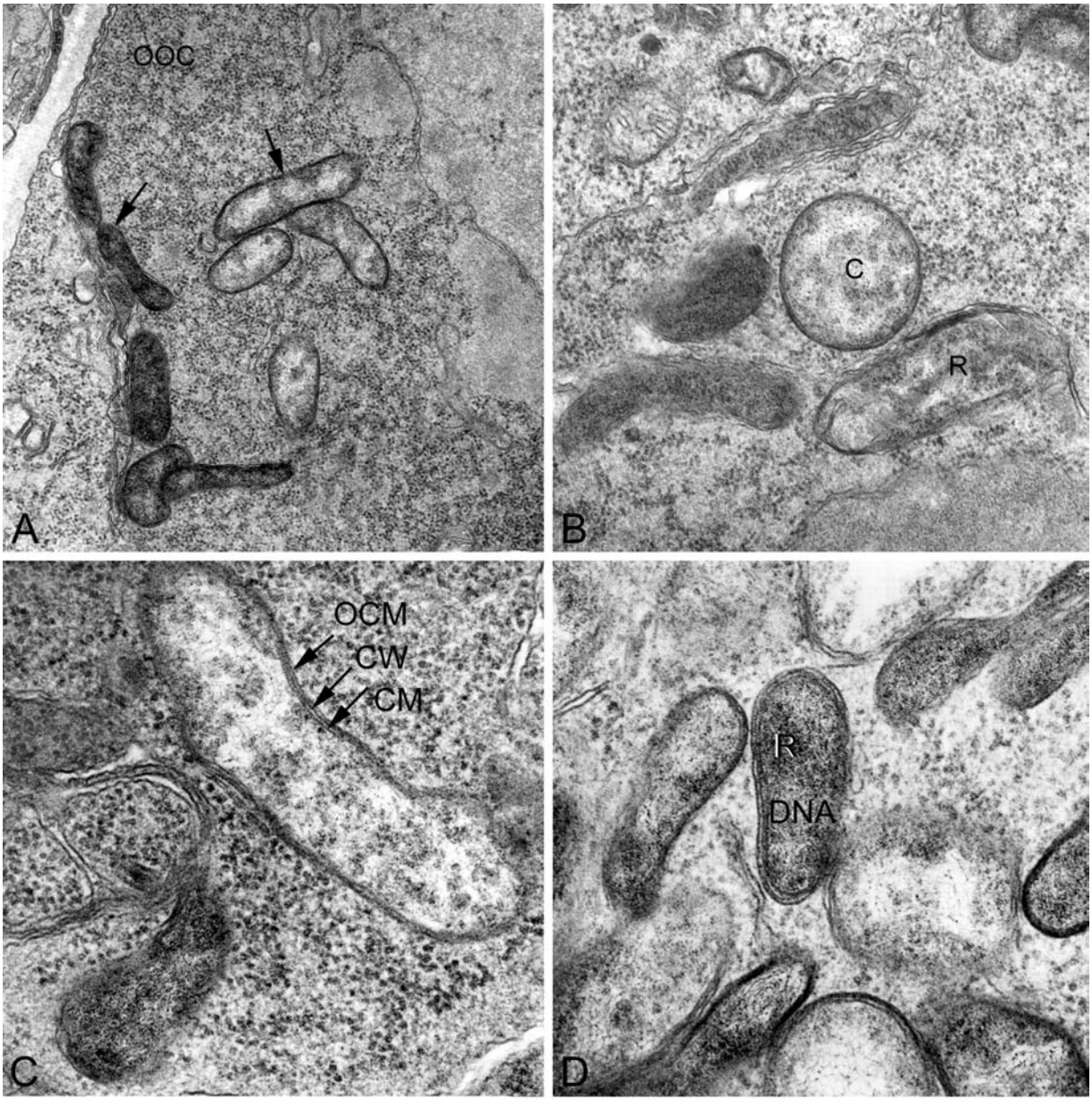


Fig. 4-8. Transmisssion electron micrographs of *Wolbachia* in the ovary of *Cx. quinquefasciatus*. **A.** The presence of *Wolbachia* (arrows) in a oocyte of *Cx. pipiens* **X 16 000**. **B.** Two forms of *Wolbachia* namely: rods (R) and cocci (C) in a oocyte of *Cx. pipiens* **X 12 000**. **C.** *Wolbachia* showing three membranes, namely: cell membrane (CM), cell wall (CW) and an outermost cell membrane (OCM) **X 40 000**. **D.** Ribosomes (R) and strands of DNA in the cytoplasm of *Wolbachia* **X 40 000**.

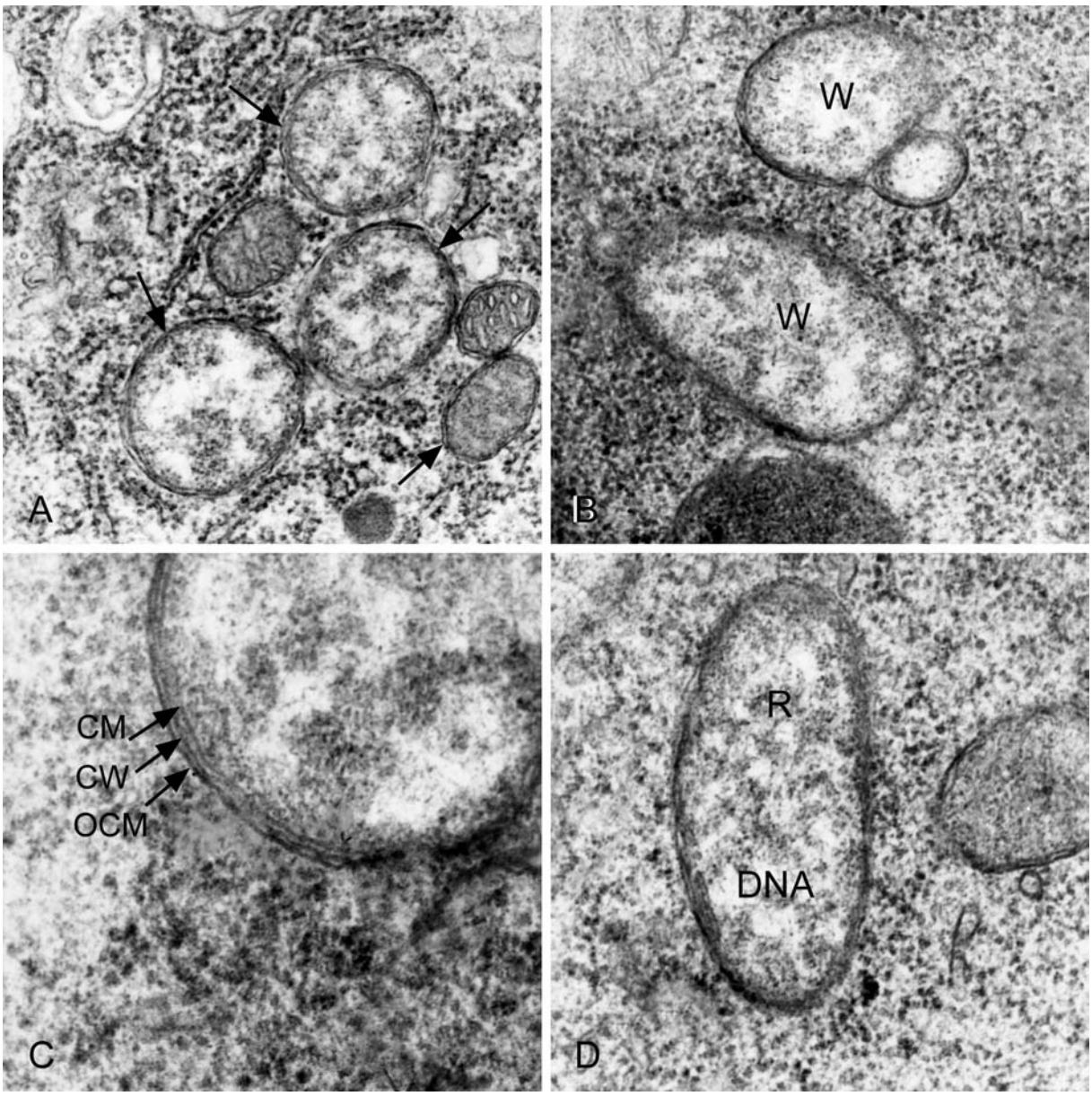


Fig. 4-9. A&B. Transmission electron micrographs of *Wolbachia* in the ovary of *D. melanogaster.* **X 32 000**, **X 40 000**, respectively (arrows and w). **C.** *Wolbachia* showing the three membranes, namely: cell membrane (CM), cell wall (CW) and an outermost cell membrane (OCM). **X 80 000**. **D.** Ribosomes (R) and strands of DNA in the cytoplasm of *Wolbachia*. **X 50 400**.

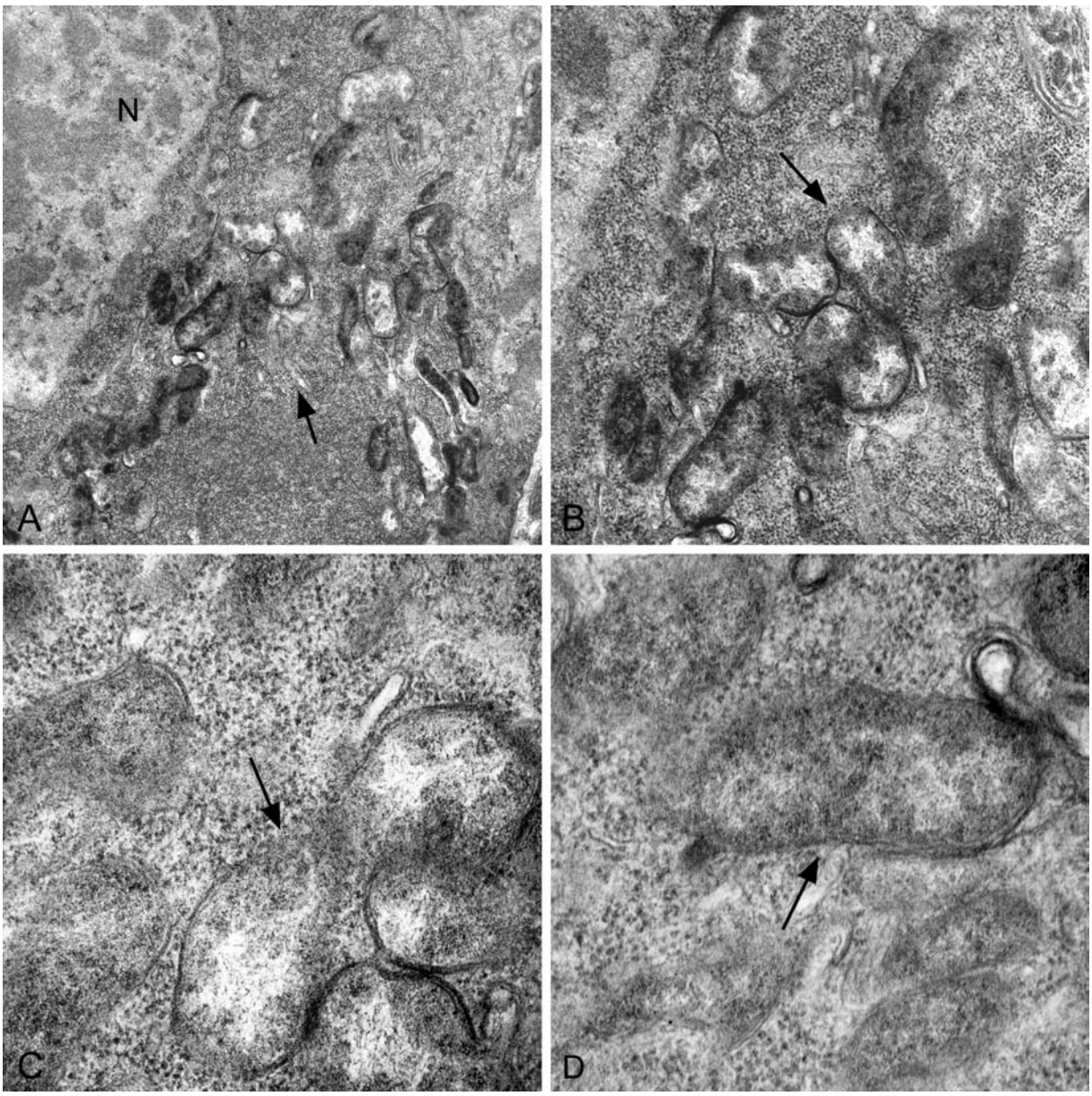


Fig. 4-10. A, B, C & D. Transmission electron micrographs of *Wolbachia* in the ovary of *Cx. pipiens* (arrows). **X 10 000**, **X 24 000**, **X 40 000**, and **X 40 000**, respectively.

4.4. DISCUSSION

The results obtained from the underground populations exhibited a high rates of *Wolbachia* infections. Under these extreme underground breeding conditions, only populations will survive which are able to reproduce in great numbers. The conditions in the underground breeding sites lead to a high selection pressure towards stenogamy, autogeny and continuous reproduction without diapause. The advantages for the reproduction of *Cx.* population in underground breeding sites are evident: 1)the constant high content of organic materials in the breeding sites which favors the larval development, 2) the absence of predacious organisms and 3) the microclimate conditions especially that of constant moderate temperatures are favourable for the development of these populations. On the other hand the lack of hosts for blood meal and the limited space for copulation result to autogeny and stenogamy (Becker et al.,1999). In my point of view, infections with *Wolbachia* are not contradictory to the selection pressure in the evolutionary process because a female carrying *Wolbachia* can mate with either infected or uninfected males and produce viable eggs, all of which are infected with *Wolbachia.* In studies on *Aedes albopictus,* it could also be shown that a *Wolbachia* superinfection is associated with both CI and increased host fecundity. In contrast to uninfected females, infected females live longer, produce more eggs, and have higher hatching rates in compatible crosses (Dobson et al., 2001).

However, when an infected male mates with an uninfected female, the tainted sperm are not able to fertilize the eggs. Meanwhile, *Wolbachia* that are present in female individuals produce an antidote that somehow restores the sperm to their full viability (Zimmer, 2001).

Furthermore, temperature has an effect on the abundance and spread of *Wolbachia.* In some arthropods, *Wolbachia* are destroyed at high temperatures (Rigaud et al., 1997). As it could be shown in experiment of spotted spider mites that were exposed to a temperature of 32+/-0.5 °C *Wolbachia* infections were eliminated after four generations (Opijnen, 1999). In the study, temperatures of more than 30 °C are frequently observed during hot summer times. This could be one of the reasons for the lower infection rates of *Wolbachia* observed in populations sampled from above surface like the population referring to accession letter B. In contrary, populations breeding in underground are not exposed to high temperature and have more constant moderate temperatures. Additionally, the effect of temperature could be the reason

why there are mixtures of infected and uninfected populations of *Wolbachia* in the field according to the exposure to sunlight (Opijnen, 1999).

It could be proven that PCR is a very reliable method in detecting the presence of *Wolbachia* infections in species of the Cx. Pipiens Complex. Infected and uninfected individuals were identified correctly and no reactions failed to amplify the *Wolbachia*-specific DNA. A comparison of the results of dot-blot assay and PCR for detecting *Wolbachia* infection in mosquitoes has documented that the dot-blot assay was not as reliable as PCR due to the fact that in some instances the dot-blot assay failed to amplify the *Wolbachia*-specific DNA (Rasgon & Scott, 1999).

Transmission electron microscopic studies further confirm the results obtained by PCR and proved that the ultrastructure of *Wolbachia* in Culex Pipiens Complex as what has been described in the literature (Clements, 1992). Furthermore, the ultrastructure of *Wolbachia* in *D. melanogaster* as a model specimen proved the presence of *Wolbachia* in species of the Culex Pipiens Complex.

5. IgG-Capture Enzyme-Linked Immunosorbent Assay (ELISA) of serum samples from Filipino patients, Cebu City, Philippines

5.1. INTRODUCTION

5.1.1. History of dengue fever

Arbovirus diseases such as dengue fever constitute an important burden to mankind in terms of morbidity and mortality (Becker & Margalit, 1993; Sang et al., 1997). The first reported epidemics of dengue fever occurred in Asia, Africa, and North America in 1779 to 1780 (Read & Ellis-Pegler, 2001). The simultaneous occurrence of outbreaks in these three continents indicates that these viruses and the mosquito vector have had a world-wide distribution in the tropics for more than 200 years (CDC, 1997).

A global pandemic of dengue began in Southeast Asia after World War II and has intensified during the last 15 years (CDC, 1997). Epidemics caused by multiple serotypes are more frequent and the geographic distribution of dengue viruses as well as the mosquito vectors have been expanded.

Globally, there are 50 million cases of dengue that are reported annually, including 500,000 hospitalizations and approximately 24,000 deaths (WHO, 1997a). In South-east Asia and in the Pacific region, reports of increased dengue fever cases were observed in Vietnam, Cambodia, Laos, Malaysia and the Philippines.

The rapid increase of population growth in the Philippines have somehow contributed to the rising dengue cases. There were 12,811 reported cases of dengue with 314 deaths in 1997. In July and August 1998, outbreaks were reported in five areas but in September, a nationwide outbreak was declared with 18 areas being declared as “dengue hotspots”. In December 15, 1998, a total of 35,648 cases with 514 deaths had been reported. From January 1 to July 27, 2001, there were 7, 697 cases of dengue, including 67 deaths. Most of the cases (53%) were males with majority belonging to ages 1-9 (DOH, 2001).

5.1.2. Description of IgG ELISA test

Serology is a useful aid in the diagnosis of dengue infections. One of the tools that aid in the diagnosis of active dengue infections is IgG capture ELISA. Active dengue infection shows a rising levels of IgG in paired sera. Anti- dengue virus IgG antibodies are produced approximately two weeks after infection and are maintained throughout in life.

In this study the commercially available enzyme-linked immunosorbent assay (ELISA) is used in diagnosing the presence of dengue infections among the Filipinos.

5.2. MATERIALS AND METHODS

5.2.1. Blood serum samples

Blood serum samples were taken from the Cebu City Medical Center Hospital, Cebu City, Philippines from January to May 1999. A total of 173 blood serum samples which consist of 108 females and 65 males suspected of having dengue infections were assayed. The ages of the patients were not identified because no available data were present in the hospital.

5.2.2. Test procedure

Blood serum samples were defrosted one night prior to the test. The microwells were removed from the foil sachet and inserted to the strip holder. Five microwells were required for the negative control while both positive and cut-off calibrator were performed in triplicates. Ten µL of patient's serum, diluted in 1:1,000 in the diluent provided, was added to each well of an assay plate containing anti-human IgG that captures the IgG in the patient's serum samples. Alternatively, 90µL of serum diluent was added to 10µL of patient's serum and 20µL of the diluted serum were added to 180µL serum diluent.

One hundred µL of diluted test, calibrator and control sera were pippeted into their respective microwells. The plates were covered and were incubated for 30 min. at 37 °C. Later, the assay plates were washed manually (6 times).

One hundred μL of HRP conjugated anti-human IgG were transferred from the antigen plate to the assay plate. These complexes were captured by dengue virus-specific IgG during an incubation period of 10 minutes at 37°C. The plates were washed and the bound complexes were visualized by adding 100 μL of tetramethylbenzidine (TMB) substrate per well.

5.2.3. Data analysis

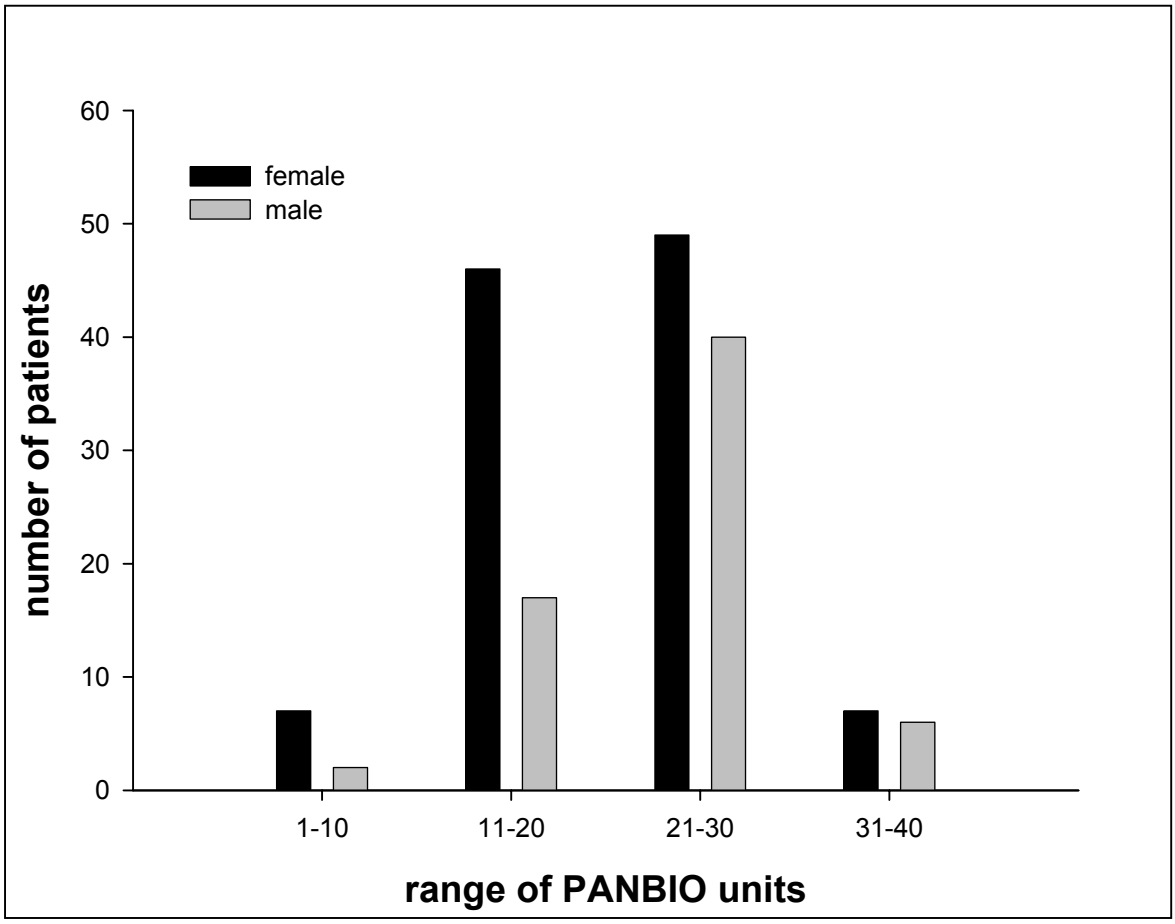
The strips were read at 450 nm with a microtiter plate reader. The clinical data were correlated with serum antibody levels. The proportions of patients with levels above the designated cut-off for the ELISA was determined. This was done by using the standard formula:

PANBIO units= absorption of sample/ absorption of cut-off x 10

Positivity was determined by comparison to reference sera containing IgG provided in the assay kit (cut-off calibrators). A positive sample was defined as having PANBIO unit of >11. Negative sample was defined as having PANBIO unit of <9 while equivocal sample (+ or -) was defined as having PANBIO unit of 9-11.

5.3. RESULTS

One hundred sixty four out of 173 (94.8%) serum samples showed positive of dengue virus. There were only 4 out of 173 (2.3%) serum samples were found negative of dengue virus. However, there were 5 (2.9%) serum samples showed equivocal or either + or - (Graph 5-1 & Appendix 5-1 to 5-7).



Graph 5-1 Number of patients infected with dengue virus.

PANBIO units:
<9= negative (-) of dengue virus
9 to 11= equivocal (+ or -)
>11= positive (+)

5.4. DISCUSSION

The results obtained from the study shows a very high percentage rate of patients that were positive or infected with dengue virus. This result is not surprising due to the fact that the blood serum samples were collected from a hospital where the poor patients were coming for treatment. These people have insufficient money for the medication. So far as they are able to tolerate the disease, they preferred staying at home and taking some herbal medications. As a result of their poverty, these people or patients came to the hospital only when they were experiencing severe symptoms.

Furthermore, the above data showed that out of 173 serum samples, 108 were females and 65 were males. The high number of females that were infected with dengue virus could be explain by the fact that females especially the housewives stayed at home most of the time. Thus, the chance of exposure to dengue vector mosquito is at high risk. In contrary, the males are seldom at home during day time when the day-biting mosquitoes are present because they are busy in their daily work, thus, the risk of having dengue infections is less.

The results obtained from this study showed higher positive in terms of blood serum samples with dengue infections compared to the previous study that was conducted in the Philippines. During the previous years, there were only 80.42% of serum samples that showed positive of IgG ELISA test (Buerano, 2000).

No vaccine is currently available for dengue fever. At present, the only method of controlling or preventing the occurrence of the virus is to combat the vector mosquitoes. Vector control is implemented using environmental management and chemical methods. Proper waste disposal, elimination of stagnant water in domestic environment and improved water storage practices such as covering the containers to prevent the access of mosquito laying eggs.

In the Philippines, health education campaigns were strengthened and dengue operation centers were established to function as screening stations. A national dengue program has never become functional, except for providing some training and education through the media. The people are now aware that the problem will get bigger as the world's population grows and poor living conditions remain in urban areas. The reasons for this dramatic global emergence of dengue/DHF as a major public health problem are complex and not well

understood. However, several important factors can be identified. First, effective mosquito control is virtually non-existent in most dengue-endemic countries. Considerable emphasis for the past 20 years has been on ultra-low-volume insecticide space sprays for adult mosquito control, a relatively ineffective approach for controlling *Ae. aegypti*. Second, major global demographic changes have occurred, the most important of which have been uncontrolled urbanization and concurrent population growth. These demographic changes have resulted in substantial housing and inadequate water, sewer, and waste management systems, all of which contribute to the increase of *Ae. aegypti* population and facilitate transmission of *Ae. aegypti* bearing disease. Third, increased travel by airplane provides the ideal mechanism for transporting dengue viruses and other pathogens. Lastly, in most countries the public health infrastructure has deteriorated. Limited financial and human resources and competing priorities have resulted in a "crisis mentality" with emphasis on implementing the so called emergency control methods in response to epidemic rather than on developing programs to prevent epidemic transmission.

Prospects for reversing the recent trend of increased epidemic activity and geographic expansion of dengue are not promising. New dengue virus strains and serotypes will continue to be introduced into many areas where the population densities of *Ae. aegypti* and *Ae. albopictus* are at high levels. With the discovery of *Bacillus thuringiensis* subsp. *israelensis* (B.t.i.) in 1976 inaugurated a new chapter in the vector control (Becker & Margalit, 1993). In recent years public health authorities have emphasized disease prevention and mosquito control through community efforts to reduce larval breeding sources. Although this approach will probably be effective in the long run, it is unlikely has an impact of disease transmission in the near future.

Although this study showed that dengue infections remain an important cause of hospitalization in the Philippines, however, the occurrence of life-threatening dengue haemorrhagic fever as has been described in several other large urban areas in Southeast Asia appears to be rare.

Furthermore, improved and pro-active laboratory-based surveillance systems that can provide early warning of an impending dengue epidemic should be recommended. An accurate and rapid method for the detection of dengue infection is important for both the clinician and the patient. The commercially available ELISA that was used in this study (PANBIO Dengue

Indirect IgG ELISA Test) is suitable for the detection of anti-dengue virus IgG antibodies in a routine clinical laboratory. At the very last, surveillance results can alert the public to take action and the physicians to diagnose as well as properly treat dengue/DHF cases.

6. Control of dengue vectors in Cebu City, Philippines

6.1. INTRODUCTION

6.1.1. Overview of dengue fever

Dengue and dengue haemorrhagic fever have emerged as the major public health problem which threaten more than three billion people in over 100 tropical and subtropical countries (WHO, 1997a). About 500,000 people are hospitalized (95% of those affected are children) and about 24,000 of deaths are reported annually. (Halstead, 1980, 1982; WHO, 1997a).

The distribution of dengue fever includes 100 endemic countries throughout America, South-east Asia, the western Pacific islands, Africa and the Eastern Mediterranean (WHO, 1997a). The classical form of dengue has been known for more than 100 years. Due to the mild clinical symptoms, it was characterized as a relatively benign disease, transmitted by a few species of mosquitoes (Becker et al., 1991). However, the clinical picture of this disease has been changed over the last decades. The incidence of dengue and the frequency of epidemics that are caused by various serotypes have increased tremendously. Thus, the clinical picture of the disease has been changed to a severe and fatal haemorrhagic form which is known as dengue haemorrhagic fever (DHF).

Since the first appearance of dengue haemorrhagic fever in Manila, Philippines in 1954, five years later in Bangkok, Thailand and in Surabaya, Indonesia in 1968, it has become presently as one of the leading causes of hospitalization and death among children and adults in the tropical regions. Dengue fever outbreaks are rising in South America and Asia. Among the affected countries, Philippines is considered as one of the high risk zones (WHO, 1997a).

The causative agent of dengue fever is an arthropod-borne virus belonging to the family Flaviviridae. There are four known serotypes of dengue: DEN-1, DEN-2, DEN-3, and DEN-4. The viruses are transmitted via the bite of the day feeding mosquitoes of the subgenus *Stegomyia*. The principal vector is *Aedes aegypti* and the secondary vector is *Aedes albopictus*. *Ae. aegypti* has been spread throughout the tropics and into susceptible human populations especially in urban areas. Once infected, a mosquito remains infective for its life span.

The infected humans circulate the virus through the blood and the mosquitoes are ingesting the viruses upon feeding on the infected individual. Humans serve as an amplifying host, though some monkeys may also serve as a source of the virus. However, female mosquitoes can also transmit the virus transovarially by passing it to the next generation (WHO, 1997b).

Dengue fever is a flu-like illness with varying symptoms. Infants often experience an undifferentiated febrile disease with skin rashes. Adults may have mild febrile symptoms but more typically experience high fever, severe headache, pain behind the eyes, skin rashes as well as muscle and joint pains. Typically, a person will develop dengue fever as a result of initial exposure to one serotype. Upon recovery, a patient develops immunity to this single serotype. During the second infection with a different serotype, the patient is prone to a greater risk of developing DHF, which is a more serious and potentially fatal disease. DHF is characterized by high fever, haemorrhagic phenomena, enlargement of the liver and circulatory failure. A sudden onset of fever is the first indication of DHF which is accompanied by facial flush and other symptoms of dengue fever. The fever persists for 2-7 days and can reach to 41°C, followed by febrile convulsions and haemorrhagic phenomena. The patient may recover and symptoms abate. But if left untreated, the patient may go into dengue schock syndrome (DSS) with rapid, weak pulse, followed by some indications of circulatory failure such as cool and blotchy skin. Without proper treatment, the patient may die within 12-24 h (WHO, 1997b).

The resurgence of dengue can be traced to a rapid urbanization, poor sewerage system and improper disposal of garbage. The persistence and numerical increase of these vectors are partly attributed to the sanitary and hygienic practices. The urbanization process which has left many households having inadequate water supply hastened the spread of the disease (Becker et al., 1991). Unfortunately, most people do not see the seriousness of the situation until they themselves become infected of the disease. Many people dispose their garbages and wastes just outside their door steps, on the streets and on vacant lots. All these improperly disposed wastes that can accumulate water during rainfall are the potential breeding sites of dengue vectors.

6.1.2. General tools in controlling dengue vectors

Vector control programs still rely to a great extent on chemicals. The annual demand amounts to more than 50,000 tons, with DDT being by far the most commonly used insecticide. Besides their life-saving benefits, insecticides have oftenly undesirable effects. For instance, vectors become resistant and the non-selectivity of the chemical oftenly causes ecological damage (Becker & Margalit, 1993).

Although intensive efforts have been done to develop a suitable vaccine against dengue, however, no vaccine is available at present. Within the scope of medical treatment, the only means of reducing the case fatality rate is early diagnosis and proper case management (Bang & Shah, 1986). The only possibility of controlling the disease is by attacking the vector (Becker et al., 1991). It is of course much cheaper to prevent an outbreak of the disease than to diagnose and to treat the cases. It is estimated for example that each DHF case costs between 50 to 200 U.S. dollar, depending on the seriousness of the case (Sumarmo & Suroso, 1987).

The main methods of controlling dengue vectors are: 1) reduction of breeding sites by means of environmental sanitation. This refers to the elimination of all non-essential water collections or receptacles that serve as breeding sites of *Aedes* species. This is the most effective method in terms of long-term reduction of the mosquito population; 2) protection of water containers, for example, by putting lids or covers to prevent egg laying of mosquitoes; 3) release of larvivorous fish as predators of larvae; 4) observation of a „Weekly Dry Day“, meaning to say that the containers are to be emptied at least once a week; 5) cleaning the containers before and after the rainy season can also contribute in reducing the mosquito populations; and lastly which is the most commonly method is by 6) space spraying, for example, with Malathion against adult mosquitoes and larviciding with Temephos (Becker et al., 1991).

Despite all of the control measures mentioned above, sustained community participation is still the strongest asset of a successful campaign. An integrated community-based approach, with volunteers from the village, is the most promising method of motivating a community to participate in vector control programmes. Women can be of vital importance, since their participation enhances the possiblity of reaching individual households and of ensuring

suatained community support (Yoon, 1987). Integration with other community projects, such as water supply, community development, sanitation programmes, child education, and income-generating activities can help in sustaining the vector control programmes on the basis of community participation.

With the alarming yearly increase of dengue fever incidence, the government agencies, non-government organizations as well as the private citizens in Cebu City, Philippines have joined their efforts to control or eradicate the dengue vectors. The efficacy of *Bacillus thuringiensis israelensis* (B.t.i.) against *Aedes* larvae was evaluated in some selected areas in Cebu City.

Cebu City (pop. 604,407; pop. density 2,162.8 indiv./per km^2), the second highly urbanized City in the Philippines is considered as a high risk zone of dengue incidence. Records of dengue fever incidences especially in densely populated areas in Cebu City showed that a high percentage of deaths among the residence particularly children between one to nine years old (DOH, 2001).

6.1.3. Overview of *Bacillus thuringiensis israelensis* (B.t.i)

The discovery of B.t.i in 1976 inaugurated a new chapter in vector control (Goldberg & Margalit, 1977; Margalit & Dean, 1985). Not only it made a breakthrough in the biological control of mosquito but it was also the first time that a new pathotype (B) which is only specific to the Nematocera had been found more than 70 years that had lapsed since the discovery of the first B.t. pathotype (A), specific to Lepidoptera. The newly isolated pathotype was characterized as serotype H-14.

The insecticidal effect of B.t.i. emanates from the parasporal body (PSB), which contains four major proteins with molecular weights of 27, 65, 128 and 135 kDa (Becker & Margalit, 1993). Neither the spores nor the living bacilli appear to be involved in the insecticidal process. The more or less spherical PSB is formed at the end of sporulation and consists of three types of protein inclusion separated by thin layers.

Tests on the single, solubilized and purified proteins have shown that each type is mosquitocidal, but alone are not as toxic as the intact PSB. This high toxicity is due to a synergistic interaction between the 25 kDa protein (proteolytic product of the 27 kDa protein) and one or more of the higher molecular weight proteins (Ibarra & Federici, 1986b; Chilcott & Ellar, 1988). The PSB is toxic to many mosquitoes. Other Nematocera are only affected at significantly higher doses. Because of this high toxicity to any of the most important vectors of human disease, B.t.i. was developed rapidly with the support of WHO in cooperation of the Special Program for Research and Training in Tropical Diseases (TDR). The extensive safety-tests and environmental impact studies was quickly put into practice (Becker & Margalit, 1993).

The following studies were conducted for the reduction of dengue vectors; 1) Peoples's knowledge, attitudes and practice for integrated community based dengue control; 2) survey of mosquito breeding sites in five selected barangays in Cebu City, Philippines and lastly; 3) control of dengue vectors by using new formulation of B.t.i.

6.2. MATERIALS AND METHODS

6.2.1. Peoples's knowledge, attitude and practice for integrated community based dengue control in Cebu City, Philippines

The study was conducted in Barangay T. Padilla which is situated just outward the old commercial street called Colon in Cebu City. It is bounded in the North by M.J. Cuenco St. and a creek serves as the west and south boundary. It is physically divided by adequate road networks and river tributaries feeding the channels. It is about 20 mins. ride from the Cebu International Air Port and 10 mins. ride from the Ayala Business Park. The total surface area is more or less 14 hectares. The population density is 857 pop/ha or 85,700 pop/sq. km. (Heikoop & Kievet, 1998).

The study was conducted from June to November, 1998. The interview in 371 households was randomized through cluster sampling. The sample size was calculated based on the number of households. The questionnaires contained questions on houshold composition including socio-economic aspects of the individuals, experiences of dengue fever and related

symptoms, people's knowledge of the symptoms, causes, transmission and prevention of the disease as well as the vector control measures on a household level.

6.2.2. Survey of mosquito breeding sites in five selected barangays in Cebu City, Philippines

Five barangays in Cebu City, Philippines were selected for the study namely: Lahug, Labangon, Gaudalupe, San Nicolas and Pardo. These barangays were chosen because of the high incidence of dengue fever during the previous years (CCHR, 1996).

The survey was conducted for a period of one month. Thirty households were randomly chosen per barangay. In each household, the specific breeding sites and their numbers were recorded, namely: 20L and 50L water containers, tin barrels, discarded tires, flower pots, tin cans, discarded plastics and other breeding sites such as leaf axils and coconut husks. The number of larvae per breeding sites were estimated using the following ranges: 0, 1-10, 11-25, 26-50 and 50 above. To determine which breeding sites have the highest in number with mosquito larvae, infestation rates were computed by using the following formula:

$$\textit{Percentage} = \frac{\text{total number of specific breeding sites with mosquito larvae}}{\text{total number of specific breeding sites surveyed}} \times 100$$

In determining the infestation rate of mosquito larvae, three indices were used, namely; House Index (HI), Container Index (CI) and Breteau Index (BI) were computed per sampling area.

$$\textit{House Index (HI)} = \frac{\text{number of houses with at least one breeding site containing larvae}}{\text{total number of houses examined}} \times 100$$

$$\textit{Container Index (CI)} = \frac{\text{number of breeding sites containing larvae}}{\text{total number of breeding sites examined}} \times 100$$

$$\textit{Breteau Index (BI)} = \frac{\text{number of breeding sites containing larvae}}{\text{total number of houses examined}}$$

6.2.3. Microbial control of dengue vectors in Cebu City, Philippines

The field study on Culinex B.t.i. tablets (Tab plus lot. no. 42-891/BD) was conducted in the indigenous tree nursery at the University of San Carlos, Talamban, Cebu City, Philippines. Fifteen plastic pails (10L) which serve as artificial breeding sites of mosquito larvae were used in the study.

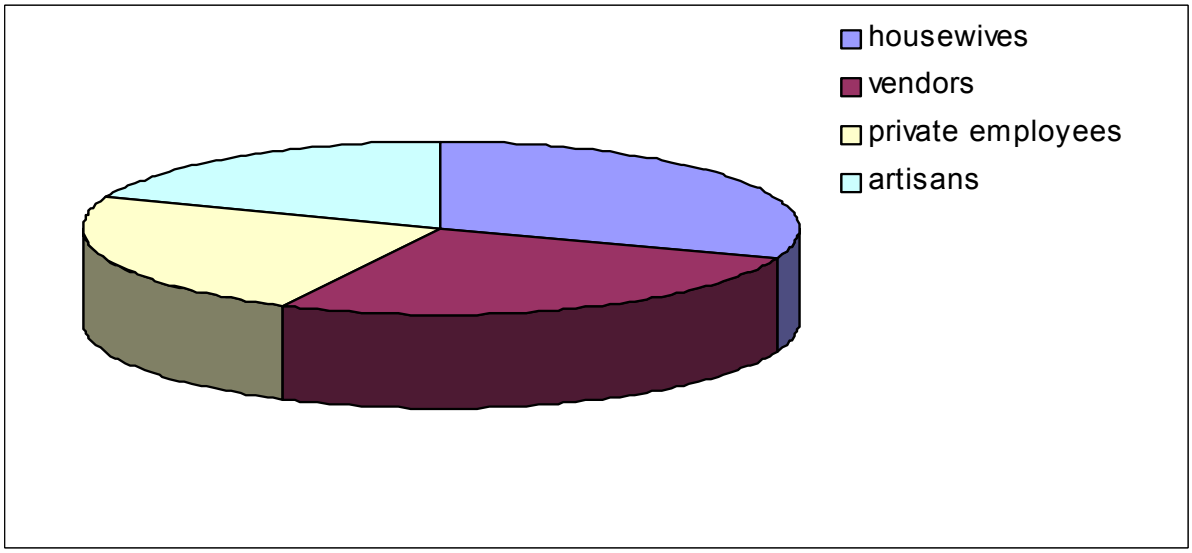
Five set-ups, each with three plastic pails (total of 15 pails) were placed randomly in selected areas at the tree nursery at the University Campus. The plastic pails were filled up with 8 liters of tap water and left under natural conditions for 14 days. The first set-up of plastic pails with corresponding numbers from 1 to 3 were treated with half of the Culinex B.t.i. tablet, 1 tablet, and control, respectively. The same procedure as in set-up 1 was applied to the remaining set-ups. To ensure the development of the eggs that are present, plastic pails were refilled with tap water to a volume of 8 liters every week. Additionally, the pails were marked with color code: white for the control, red for the treatment of half of the Culinex B.t.i. tablet and green for the treatment of one Culinex B.t.i. tablet. The efficacy of Culinex B.t.i. tablets were monitored on a daily basis during the first week of application and on a weekly basis for the continuous week until the mortality rate of less than 50% was observed. First instar larvae were not considered in the evaluation.

6.3. RESULTS

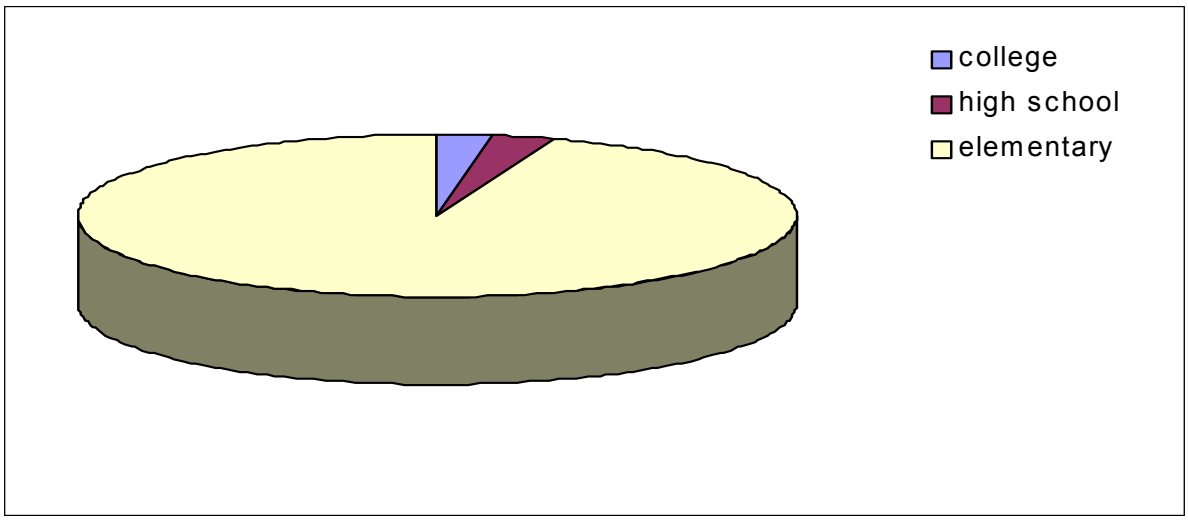
6.3.1. People's knowledge, attitudes and practice for integrated community-based dengue control in Cebu City, Philippines

The average age of the respondents was 38 years old. The respondents were 79.78% females and 20.22% males. The average number of persons in a household is six. The average number of children was three. Sixty three percent of the population were children below 15 years old and the average years of residence was 22 years. The most frequently reported occupations, educational attainments and the location of the houses as well as the type of housing description that were surveyed are summarized in Graphs and Appendix 6-1, 2, 3, and 4 correspondingly. Furthermore, the sources of water among the househols are summarized in Graph & Appendix 6-5. The drainage of 66.85% of the houses were open canals, 27% were partially closed canal and 5.66% unspecified.

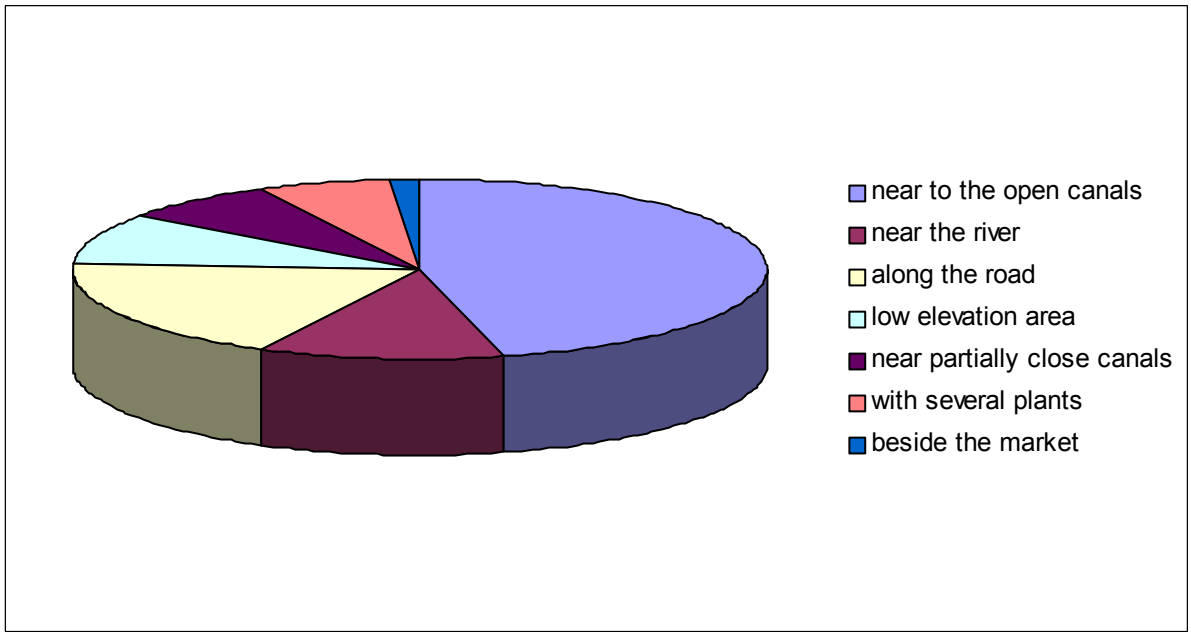
One hundred sixty nine out of 371 (45.55%) of the sample population had experienced general febrile conditions during the last six months before the interview. However, only 11.05% having reported suffered from dengue during the same period of time. Most of the respondents had knowldege on the key symptoms of dengue fever as well as 68.73% of the interviewees were aware that the disease is transmitted by mosquitoes (Graph & Appendix 6-6). However, only 4.31% of the respondents knew that a virus was the cause of the disease.



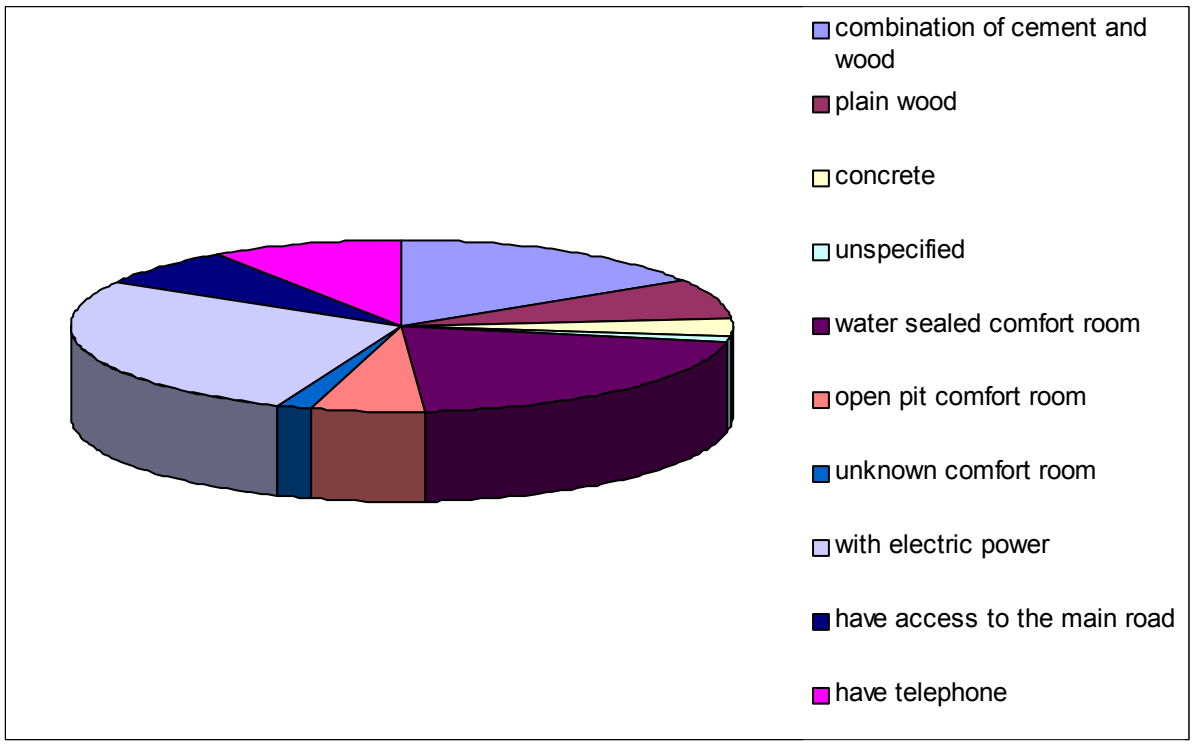
Graph 6-1. Occupations of the respondents.



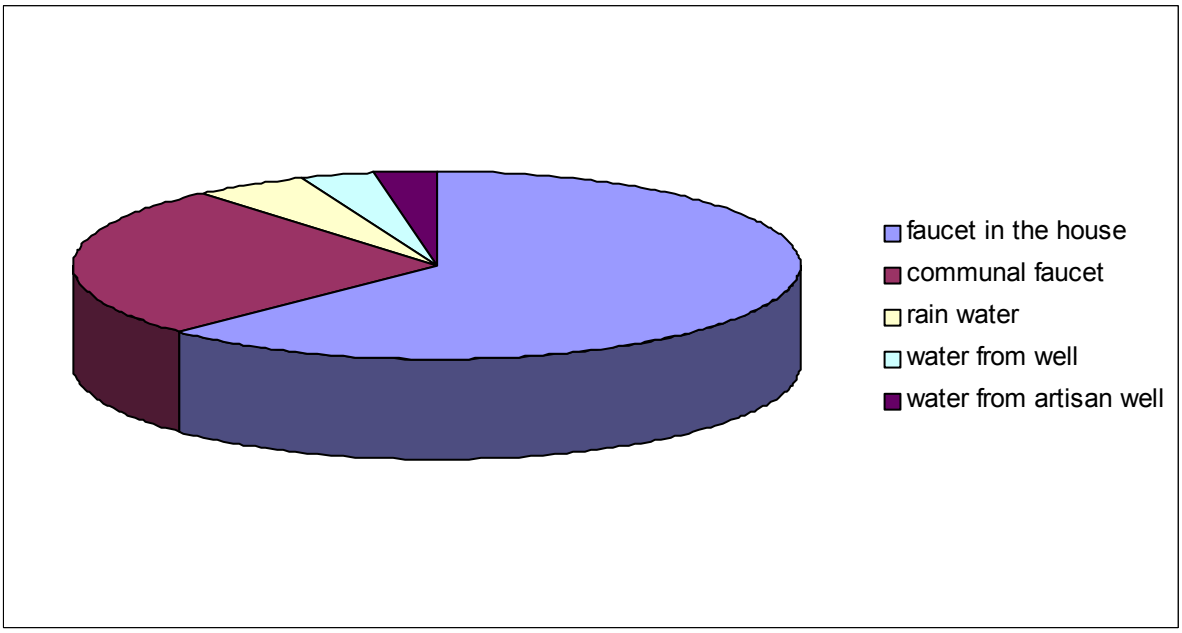
Graph 6-2. Educational attainment of the respondents.



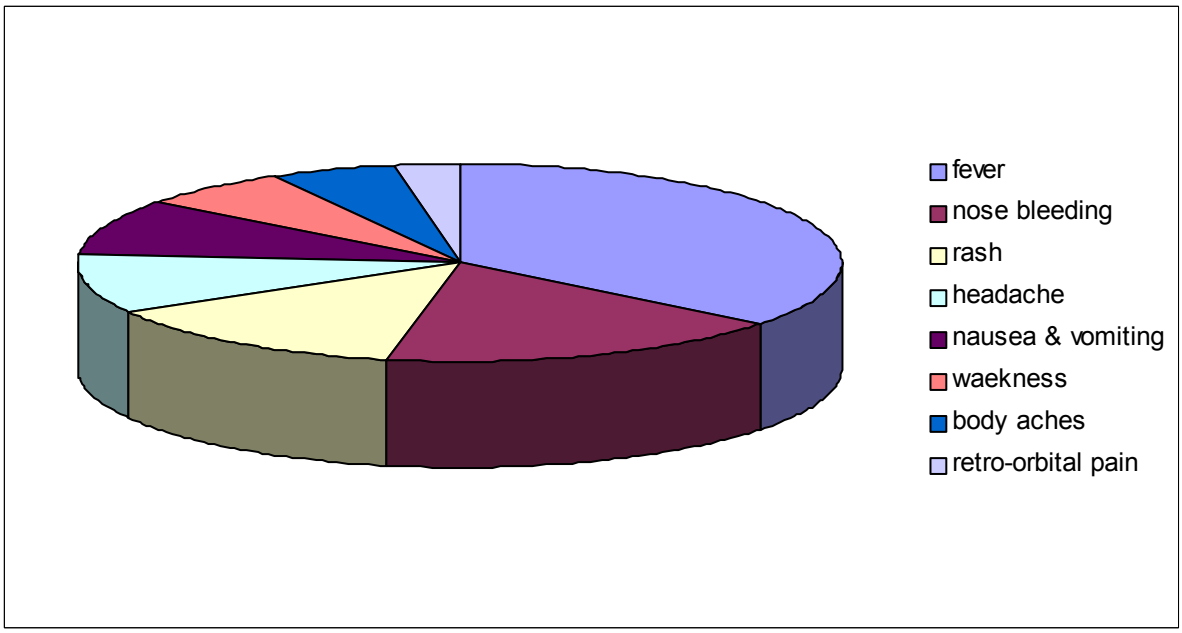
Graph 6-3. Location of the houses that were surveyed.



Graph 6-4. House description of the respondents.

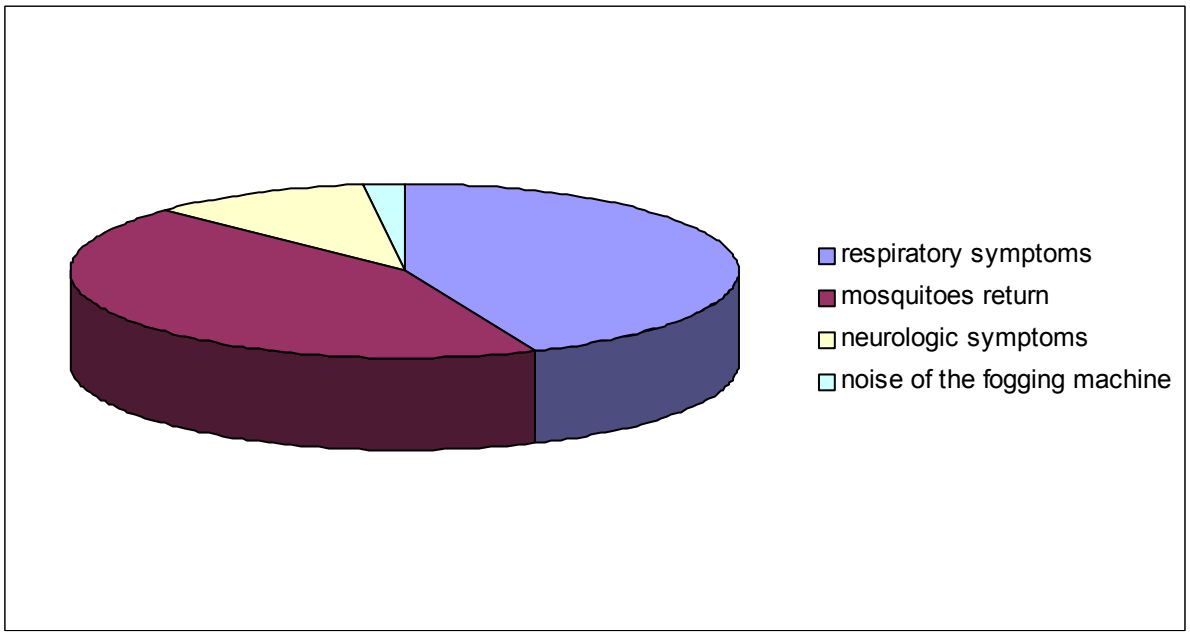


Graph 6-5. Sources of water among the households.

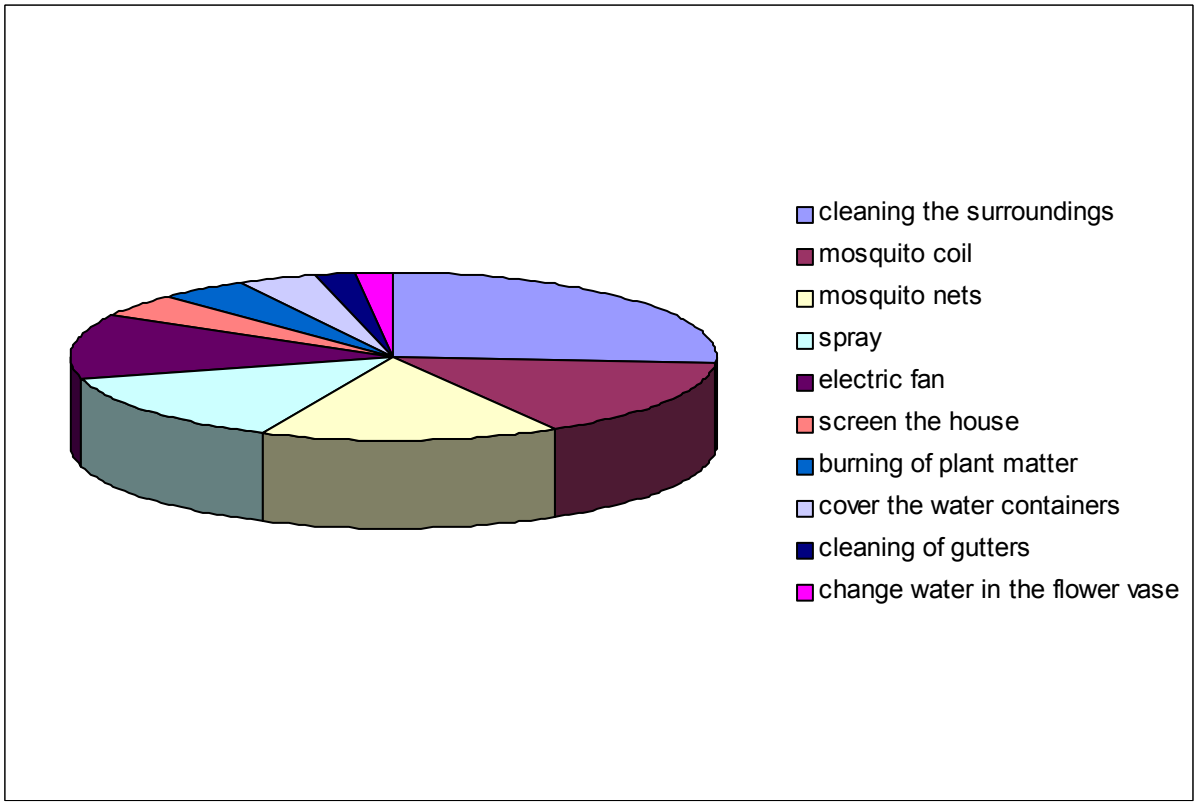


Graph 6-6. People's knowledge on the symptoms of dengue fever.

About half (52.29%) of the respondents were aware of the potential danger of mosquito breeding sites. The people were aware of fogging being done in their community. There were 48.52% of the respondents gave their negative comments on fogging. Their complaints are summarized in Graph and Appendix 6-7. Nevertheless, there were 67.65% agree that fogging should be done in the barangays. Vector control measures at the household level reported by the respondents are summarized in Graph and Appendix 6-8.



Graph 6-7. People's complains on fogging.



Graph 6-8. Vector control measures at the household level.

Almost half of the respondents were aware of the barangay initiated anti-dengue drive. However, only 47.98% of the respondents that were willing to attend the mosquito control drives and 55.53% were willing to participate in the barangay clean-up drive.

6.2.3. Survey of mosquito breeding sites in five selected barangays in Cebu City, Philippines

As shown in Table 6-9, out of 489 specific breeding sites surveyed, 142 (29.4%) breeding sites were found to have mosquito larvae. In terms of percentage of the specific breeding sites with mosquito larvae, discarded tires had the highest with 50 out of 72 (69.44%). Next in rank is discarded plastics with 13 out of 19 (68.42%). Other breeding sites such as leaf axils, coconut husks and flower pots were next in rank with above 50%.

Table 6-9. Different types of mosquito-breeding sites and their larval infestation in five selected barangays.

Breeding sites	**number of larvae**				**total breeding sites**		**total sites surveyed**	**% of sites w/ larvae**
	1-10	11-25	26-50	50 and above	w/ larvae	w/out larvae		
water container (up to 20L)	4	3	0	0	7	188	195	3.59
water container (up to 50L)	0	0	1	0	1	19	20	5.0
barrels	12	4	4	2	22	56	78	28.2
discarded tires	22	11	9	8	50	22	72	69.44
flower pots	6	1	0	1	8	8	16	50
tin cans	8	1	0	0	9	17	26	38.46
discarded plastics	6	1	2	4	13	6	19	68.42
other breeding sites (e.g. leaf axils, etc.)	16	5	8	3	32	31	63	50.79
Total					142	347	489	
Percentage (%)					70.96	29.04	100	

Among the breeding sites with the least number of larvae present were the 20L water containers with 3.9% or 7 out of the 195 surveyed and 50L water containers with only 5% or 1 out of 20 that were surveyed. Although most of these containers were left uncovered, mosquito eggs cannot develop into larvae because the water were constantly used for daily needs.

As to the most number of houses infested with mosquito larvae (Table 6-10), barangay Lahug rank first with a house index of 93.33% followed by Guadalupe (56.66%), San Nicolas

(43.33%), Labangon (36.66%) and the lowest is Pardo (33.33%). For the container index, a similar ranking resulted in Lahug as the highest (55.73%) and Pardo being the lowest (14.44%). Breteau index was again high in Lahug and low in Pardo (Table 6-11).

Table 6-10. Number of houses per barangay with breeding sites containing larvae

Barangay	No. of houses w/ breeding sites containing larvae	House index
Guadalupe	17	56.66
Lahug	11	93.33
San nicolas	28	43.33
Labangon	10	36.66
Pardo	13	33.33

Table 6-11. House index, container index and breteau index values in each barangay.

Barangay	House index	Container index	Breteau index
Guadalupe	56.66	29.89	90
Lahug	93.33	55.37	230
San Nicolas	43.33	25.27	83.33
Labangon	36.66	15	56.66
Pardo	33.33	14.44	50.0

6.3.3. Microbial control of dengue vectors

After the first week of the experimental set-up, it was observed that there was absolutely no infestation of larvae, neither with eggs nor with larvae of mosquitoes in the plastic pails. However, during the second week of experimental set-up, it was observed that each pail was infested with at least 20 larvae in various stages as well as eggs were present.

The number of larvae that were observed at different duration of times by the application of Culinex B.t.i. tablets in different dosages are summarized in Table 6-12. During the first week of monitoring (after 1 week of tablets application), it was observed that both larvae that were treated with Culinex B.t.i. tablets were already dead.

During the second week after the application, it was also observed that there was no larvae present in the treated pails. However, during the third week after the application, some live larvae were observed in the treated pails. During the fourth week after the application, it was observed that the efficacy of Culinex B.t.i. tablets was becoming less effective due to the presence of more larvae in the treated pails. On the fifth week after the application, the survey was terminated because it was observed that the B.t.i tablets were no longer effective due to the infestations of larvae (3^{rd} & 4^{th} instar).

Table 6-12. The efficacy of various B.t.i. tablet dosages against mosquito larvae.

Tablet dosage/set-up	**No. of larvae**			
	1^{st} week	**2^{nd} week**	**3^{rd} week**	**4^{th} week**
Set-up 1				
½ tablet	0	0	0	0
1 tablet	0	0	0	3
control	10	9	5	40
Set-up 2				
½ tablet	0	0	0	1
1 tablet	0	0	0	27
control	0	7	4	30
Set-up 3				
½ tablet	0	0	0	5
1 tablet	0	0	0	30
control	8	13	10	25
Set-up 4				
½ tablet	0	0	5	50
1 tablet	0	0	0	50
control	12	21	4	10
Set-up 5				
½ tablet	0	0	38	*
1 tablet	0	0	10	*
control	25	30	28	*

* termination of the survey due to larval infestations in all treated pails.

6.4. DISCUSSIONS

Knowledge, attitudes and practice towards dengue were very limited. This may be due to the fact that dengue in endemic areas is often clinically nonspecific especially in children and the people's limited knowledge regarding the symptoms (Gubler, 1998). Many of the interviewees (64.96%) mentioned fever, however, only 9.43% reported the typical symptoms of body pains and 17.79% reported headache. A few interviewees confounded signs of dengue with those of rabies and cholera because there were competing information compaign in the previous year of the Department of Health in the media about rabies, cholera as well as dengue.

This fragmentary knowledge about some aspects of the disease, transmission and prevention without an understanding of the context and the whole framework maybe one of the reasons why people do not follow the instruction to eliminate the breeding sites. To address this problem, there is really a need to carry out information, education and communication activities systematically. Education activities could be carried out through the following: 1) social mobilization to be organized by the community; 2) interpersonal communication like women's group and lastly; 3) mass media print, broadcast, radio, special limited media like vedio.

The importance of identifying the breeding sites of mosquito larvae through periodic surveys and monitoring cannot be over-emphasized. A continued localized mosquito surveys can provide a baseline knowledge for an effective health services to combat not only dengue but also other mosquito related diseases nationwide.

Results of the study showed that the abundance of breeding sites and the rate of larval infestations are closely correlated with densely populated areas. Barangays of Lahug and Guadalupe are the most densely populated among the five barangays surveyed, also had the highest number of breeding sites surveyed and a high larval infestation rates.

Likewise, most of the breeding sites that were identified in the study are the ones classified as artificial breeding sites usually provided by the people itself. Thus, the breeding sites of mosquitoes can be controlled and prevented by the local community themselves by strictly following proper sanitation and hygienic practices.

The demand of B.t.i. is increasing worldwide; about 1,000 tons of B.t.i. preparations are now being used annually (Becker et al., 1993). So far there have been no negative effects that are reported. B.t.i. is a promising tool in the fight against dengue fever and other nosquito-borne diseases. In suitable formulations, this microbial agent is a useful supplement or replacement for broad spectrum chemicals. Furtheremore, B.t. *israelensis* offers an ecological defensible compromise between the desire of man to protect himself from troublesome mosquitoes and the requirement of current environmental policies to protect sensitive ecosystems by the use of non-selective methods (Becker et al., 1993).

7. SUMMARY

Mosquitoes are threatening the human population as vectors of deliberating diseases such as malaria, dengue, yellow and west nile fever as well as lymphatic filariasis. The knowledge on the biology of mosquitoes, pathogens and parasites transmitted by them is essential for a better control of both the insects and diseases they are transmitting. This dissertation aims to increase the knowledge on the biology of vector mosquitoes as well as the pathogens. The research includes the following topics, namely: 1) protein electrophoretic studies on mosquitoes; 2) studies on ovaries of mosquitoes using light, transmission and scanning electron microscopy; 3) molecular and electron micrsocopic identification of *Wolbachia* sp. in populations of the Culex Pipiens Complex from the Upper Rhine Valley, Germany, and Cebu City, Philippines; 4) assessment of dengue viruses by IgG-Capture Enzyme-Linked Immunosorbent assay (ELISA) of serum samples from Filipino patients, Cebu City, Philippines; and 5) the efficacy of tablets based on *Bacillus thuringiensis israelensis* (B.t.i.) against the larvae of dengue fever vector, *Aedes aegypti*.

A total of 668 mosquito samples which include the populations of *Culex p. pipiens* from Germany, *Culex p. quinquefasciatus* from the Philippines and USA as well as *Culex peus* from the USA were investigated by using protein electrophoresis. Ten enzymes were studied. The highest average polymorphism and heterozygosity were observed in the population of *Culex p. pipiens*, whereas, *Culex p. quinquefasciatus* from the USA had the lowest average of polymorphism. *Culex p. quinquefasciatus* from the Philippines had the lowest average of heterozygosity. The sample populations of *Culex* were in Hardy-Weinberg equilibrium, except the population of *Culex p. quinquefasciatus* from the USA which showed significant deviation at the locus GPI. Furthermore, the populations of *Culex p. pipiens* and *Culex p. quinquefasciatus* from the Philippines and USA, showed a very low genetic distances.

The ultra-structure of the mosquito ovaries was studied using light, transmission and scanning electron microscopy. The morphology is compared for the identification of *Wolbachia* infections in the mosquito populations. Ovaries of mosquitoes with and without a blood meal were studied. Transmission electron microscopy of an ovary with a blood meal revealed some prominent structural changes. One of the striking features observed occurred in the ovarian follicles or epithelial follicles. Prominent changes include the development of large inter-cellular spaces and decrease in desmosomal connections as well as the development of pits or vesicular bodies.

The presence of *Wolbachia* was proven by using Polymerase Chain Reaction (PCR) as well as electron microscopy. Two primers were used in the PCR assays, namely: Primer set 1 which amplified a 0.9 kb fragment from *Wolbachia* 16S rDNA and is specific to *Wolbachia.* Primer set 2 is universal for insect mitochondrial DNA. Underground populations showed an infection rate of 100% compared to populations from the above ground surface, where a lower infection rate of 10% was observed. Furthermore, the presence of *Wolbachia* was proven by using transmission electron microscopy. The bacteria exhibited the typical morphology of *Wolbachia* with three cell membranes comprising the plasma membrane, cell wall and an outermost membrane which is thought to be of host origin .

A total of 173 blood serum samples from Cebu City, Medical Center, Philippines were assayed for the presence of dengue viruses by using ELISA test. One hundred sixty four out of 173 (94.83%) serum samples showed positive for dengue viruses. Four out of 173 serum samples (2.3%) were negative for dengue viruses and 5 (2.9%) showed equivocal (either + or -). The high percentage rate of dengue virus infections observed is not surprising as blood serum samples were obtained from a hospital where poor patients were coming for treatment.

The study on dengue vectors was conducted based on the following: 1) survey on people's knowledge, attitude and practice for integrated community-based dengue control; 2) survey of mosquito breeding sites in five selected barangays in Cebu City, Philippines; and 3) microbial control of dengue vectors in Cebu City, Philippines. The results of the study showed that the knowledge, attitude and practice towards dengue fever were very limited. Many of those interviewed (64.96%) mentioned fever, however, only 9.43% reported the typical symptoms of body pains and 17.79% reported headache.

There were 142 (29.4%) out of 489 of the surveyed breeding sites that were found to have mosquito larvae. Among the infested breeding sites, discarded tires had the highest infestation rate with 50 out of 72 (69.44%). Next in rank were discarded plasticwares with 13 out of 19 (68.42%). Other breeding sites such as leaf axils, coconut husks and flower pots were next in rank having 50% above.

The efficacy of Culinex B.t.i. tablets in various dosages was tested against the larvae of *Aedes aegypti* in Cebu City, Philippines. One hundred percent mortality rate was observed during

the first week after the application of the tablets. During the third week, the mortality rate was about 78%. However, during the fourth week, no significant mortality rate was observed.

This research work should support the implementation of environmentally safe mosquito control tools in order to improve the life quality and health of humans.

8. LITERATURE CITED

Anderson, E. 1962. The ovary of *Periplaneta americana*. *Abstracts of the 2nd Annual Meeting of the American Society for Cell Biology*, San Francisco. 2.

Anderson, W. A. and Spielman, A. 1971. Permeability of the ovarian follicles of *Aedes aegypti* mosquitoes. *J. Cell Biol*. 50: 201-221.

Ayala, F. J. 1975. Genetic differentiation during the speciation process. *Evol. Biol*. 8:1-78.

Bandi, C., Anderson, T. G., Genchi, C. and Blaxter, M. L. 1998. Phylogeny of *Wolbachia* in filarial nematodes. *Proc R Soc Lond Biol Sci*. 265: 2407-2413.

Bang, Y. H. and Shah, N. K. 1986. Regional review of DHF situation and control of *Aedes aegypti* in South-east Asia. *Dengue Newsletter* Vol.12.

Barr, A.R. 1957. The distance of *Culex p. pipiens* and *Cx. p. quinquefasciatus* in North America. *Am. J. Trop. Med. Hyg*. 6: 153-165.

Barr, A. R. 1958. The Mosquitoes of Minnesota (Diptera: Culicidae). *Univ. Minn. Agric. Exp. Stn. Tech. Bull*. 228, 154pp.

Barr, A. R. and Azawi, A. 1958. Notes on the oviposition and the hatching of eggs of *Aedes* and *Psorophora* mosquitoes (Diptera: Culicidae). *Univ. Kans. Sci. Bull*. 7: 95-99.

Barr, A. R. 1981. Cytogenetics and genetics of vectors. *Proceedings of a Symposium of the XVIth International Congress of Entomology.* 123-136 pp.

Barr, A. R. 1982. Symbiont control of reproduction in *Culex pipiens*. *In Recent Developments in the Genetics of Insect Disease Vectors* (eds W. W. M. Steiner, W. J. Tabachnick, K. S. Rai and S. Narang), Stipes Publishing Co., Champaign, Illinois, 153-158 pp.

Beach, R. 1978. The required day number and timely induction of diapause in geographic strains of the mosquito *Aedes atropalpus*. *J. Insect. Physiol.* 24: 448-455.

Beaty, B. J. and Marquardt, W. C. 1996. *The biology of disease vectors*. University Press of Colorado, Colorado, USA, 632 pp.

Becker, N. 1989a. Life strategies of mosquitoes as an adaptation to their habitats. *Bull. Soc. Vector Ecol*. 14(1): 6-25.

Becker, N. 1989b. Biologische Stechmückenbekämpfung am Oberrhein. in: Biologischer Pflanzenschutz. Schriftenreihe d. Bundesministers für Ernährung, Landwirtschaft und Forsten. Angewandte Wissenschaft, Landwirtschaftsverlag GmbH, Münster-Hiltrup, Heft 344, 145-171 pp.

Becker, N., Djakaria, S., Kaiser, A., Zulhasril, O. and Ludwig, H. W. 1991. Efficacy of a new tablet formulation of an asporogenous strain of *Bacillus thuringiensis israelensis* against larvae of *Aedes aegypti*. *Bull. Soc. Vector Ecol*. 16(1): 176-182.

Becker, N. and Margalit, J. 1993. Use of *Bacillus thuringiensis israelensis* against mosquitoes and blackflies. *Bacillus thuringiensis*, *An Environmental Biopesticide: Theory and Practice*. John Wiley & Sons Ltd. 147-170pp.

Becker, N., Jöst, A., Storch, V. and Weitzel, T. 1999. Exploiting the biology of urban mosquitoes for their control. *Proceedings of the 3rd International Conference on Urban Pests*. Graficke zavody Hronov, Czech Republic. 425-429pp.

Becker, N., Petric, D., Zgomba, M., Boase, C., Dahl, C., Lane, J. and Kaiser, A. 2003. *Mosquitoes and their control*. Kluver Academic/ Plenum Publishers. New York, Boston, Dordrecht, London, Moscow. 497pp.

Beckett, E. B., Boothroyd, B. and Macdonald, W. W. 1978. A light and electron microscope study of rickettsia-like organisms in the ovaries of mosquitoes of the *Aedes scutellaris* group. *Ann Trop Med Parasitol*. 72: 277-283.

Belkin, J. N. 1962. *The mosquitoes of the South Pacific (Diptera: Culicidae).*University of California Press, Berkeley and Los Angeles. Vol. 1.

Belkin, J. N., Schick, R. X. and Heinemann, S. J. 1966. Mosquito studies (Diptera: Culicidae). VI. Mosquitoes originally described from North America. *Contrib. Am. Entomol. Inst*. 1: 1-39.

Bertram, D. S. and Bird, R. G. 1961. Studies on mosquito-borne viruses in their vectors; the nocturnal fine structure of the midgut epithelium of the adult female *Aedes aegypti* (L.) and the functional significance of its modification following a blood meal. *Trans. R. Soc. Trop. Med. Hyg*. 55: 404-423.

Bouchon, D., Rigaud, T. and Juchault, P. 1998. Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. *Proc R Soc Lond B Biol Sci* . 265: 1081-1090.

Bourtzis, K., Pettigrew, M. M. and O'Neill, S. L. 2000. *Wolbachia* neither induces nor suppresses transcripts encoding anti-microbial peptides. *Insect Mol. Biol.* 9(6): 635-639.

Brambell, F. W. R. and Hemmings, W. A. 1954. Active transport through embryonic membranes. *Symp. Soc. Exp. Biol*. 8: 476.

Brandt, P. W. 1958. A study of the mechanism of pinocytosis. *Exp. Cell Research*. 15: 300.

Bruce-Chwatt, L. J. and de Zulueta, J. 1980. *The rise and fall of malaria in Europe*. A historico - epidemiological study. University Press, Oxfort. 240 pp.

Brust, R. A. and Costello, R. A. 1969. Mosquitoes of Manitoba. II. The effect of storage temperature and relative humidity on hatching of eggs of *Aedes vexans* and *Aedes abserratus* (Diptera: Culicidae). *Can. Entomol.* 101: 1285-1291.

Brust, R. A. and Munstermann, L. E. 1992. Morphological and genetic characterization of the Aedes (Ochlerotatus) Communis Complex (Diptera: Culicidae) in North America. *Ann. Entomol. Soc. Am*. 85: 1-10.

Bullini, L. and Coluzzi, M. 1972a. Electrophoretic studies on gene enzyme systems in mosquitoes (Diptera: Culicidae). *Parassitologia* 15: 221-246.

Bullini, L. and Coluzzi, M. 1972 b. Natural selection and genetic drift in protein polymorphism. *Nature* 239: 160-161.

Bullini, L. and Coluzzi, M. 1973. Electrophoretic studies on gene-enzyme systems in mosquitoes (Diptera: Culicidae). *Parassitologia* 15: 221-248.

Bullini, L. and Coluzzi, M. 1978. Applied and theoretical significance of electrophoretic studies in mosquitoes (Diptera: Culicidae). *Parassitologia* 20: 7-21.

Buerano, C., Ibraham, I., Contretras, R., Hasebe, F., Matias, R., Natividad, F. and Igarashi, A. 2000. IgM-capture ELISA of serum samples collected from Filipino dengue patients. *Southeast Asian J Trop Med Public Health* 31(3): 524-529.

Caro, L. G. and van Tubergen, R. P. 1962. High resolution autoradiography. *J. Cell Biol*. 15: 173.

Cebu City Health Record (CCHR). 1996. Cebu City, Philippines. 112pp.

Center for Disease Control (CDC). 1997. Information on dengue fever and dengue haemmorhagic fever. Morbidity and Mortality Weekly Report. 43:6.

Chargaff, E. 1942. The formation of the phosphorous compounds in egg yolk. *J. Biol. Chem*. 142: 505.

Chilcott, C. N. and Ellar, D. J. 1988. Comparative toxicity of *Bacillus thuringiensis* var. *israelensis* crystal proteins in vivo and in vitro. *J. Gen. Microbiol*. 134: 2551-2558.

Christophers, S. R. 1961. *Aedes aegypti* L. *its life history, bionomics and structure.* London, Cambridge University Press, 676pp.

Clements, A. N. 1992. Rickettsiae in mosquitoes*. The Biology of mosquitoes* London: Chapman and Hall. 509 pp.

Cockran, W. G. 1954. Some methods for strengthening the common chi-square tests. *Biometrics* 10: 417-451.

Crisp, D. J. 1978. The Hardy-Weinberg test-a correction. *Mar. Biol*. 46: 181-183.

Curtis, C. F. and Sinkins, S. P. 1998. *Wolbachia* as a possible means of driving genes into populations. *Parasitology* 116: 111-115.

Department of Health (DOH). 2001. Cebu City, Philippines. 98pp.

Dobson S. L, Marsland E. J. and Rattanadechakul, W. 2001. *Wolbachia*-induced cytoplasmic incompatibility in single and superinfected *Aedes albopictus* (Diptera: Culicidae). *J Med Entomol* 38(3): 382-387.

Dobzhansky, T. and Levene, H. 1948. Genetics of natural populations. XVII: Proof of operation of natural selection in wild populations of *Drosophila pseudoobscura*. Genetics, Princeton. 33: 537-547.

Eldridge, B. F. and Edman, J. D. 2000. *Medical Entomology*. Kluwer Academic Publishers, Dordrecht, Boston, London. 659 pp.

Emigh, T. H. 1980. A comparison of tests for Hardy-Weinberg equilibrium. *Biometrics*. 36: 627-642.

Evans, P. G. H. 1987. Electrophoretic variability of gene products. *Academic Press* 106-154.

Farid, H. A., Gad, A. M. and Spielman, A. 1991. Genetic similarity among Egyptian populations of *Culex pipiens* (Diptera: Culicidae). *J. Med. Entomol*. 28(2): 198-204.

Felsenstein, J. 2002. Neighbor- Joining and UPGMA methods. University of Washington. Seattle, USA.

Ferguson, A. 1980. *Biochemical systematics and evolution*. Blackie, Glasgow. 477pp.

Francki, R. I. B., Fauquet, C. M., Knudson, D. L. and Brown, F. 1991. Classification and nomenclature of viruses. Fifth report of the International Committee on Taxonomy of Viruses. *Archives of Virology*, Suppl. 2, Springer Verlag, Wien, 452 pp.Garnham, P.C.C., 1966. Malaria Parasites and other Haemosporidia. *Blackwell Scientific Publications*, Oxford, 1114 pp.

Garnett, P. and French, W. L. 1971. A genetic study of an esterase in *Culex pipiens quinquefasciatus*. *Mosquito News* 31: 379-386.

Gratz, N. G. 1999. Emerging and resurging vector-borne diseases. *Ann. Rev. Entomol*. 44: 51-75.

Gillett, J. D. 1955. Variation in the hatching-response of *Aedes* eggs. *Bull. Ent. Res*. 46: 241-253.

Gillett, J. D. 1983. Abdominal pulses in newly emerged mosquitoes *Aedes aegypti*. *Mosq. News* 43: 359-361.

Goldberg, L. H. and Margalit, J. 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata, Culex univattatus, Aedes aegypti* and *Culex pipiens*. *Mosq. News* 37: 355-358.

Gubler D. J., Novak, R. and Mitchell, C. J. 1982. Arthropod vector competence: epidemiological, genetic and biological considerations. *In Recent Developments in the Genetics of Insect Disease Vectors*. Stipes Publishing Co., Champaigne, Illinois. 450pp.

Gubler, D. J. 1998. Dengue and dengue haemorrhagic fever. *Clinical Microbiology Reviews*. 11(3): 480-496.

Haldene, M. B. S. 1954. An exact test for randomness of mating. *J. Genet.* 52: 631-635.

Halstead, S. B. 1980. Dengue haemorrhagic fever - a public health problem and a field for research. *Bull. WHO* 58(1): 1- 21.

Halstead, S. B. 1982. WHO fights dengue haemorrhagic fever. *WHO Chron*. 38(2): 65-67.

Halstead, S. B. 1992. The XXth century dengue pandemic: need for surveillance and research. *World Health Stat. Q*. 45: 292-298.

Hardy, G. H. 1908. Mendelian proportions in a mixed population. *Science* 28.

Harris, H. and Hopkinson, D. A. 1976. *Handbook of enzyme electrophoresis in human genetics*. North-Holland Publ. Co., Amsterdam, Oxford.

Heikoop, R., and de Kievet, R. 1998. Urban drainage research in a squatter settlement. Unpublished.

Hertig, M. 1936. The rickettsia, *Wolbachia pipientis* and associated inclusions of the mosquito, *Culex pipiens*. *Parasitology* 28: 453-490.

Hoerauf, A., Volkmann, L., Hamelmann, C., Adjei, O., Autenrieth, L. B., Fleischer, B. and Buttner, D. W. 2000. Endosymbiotic bacteria in worms as targets for a novel chemotherapy in filariasis. *Lancet* 355: 1242-1243.

Holden, P. R., Jones, P. and Brookfield, J. F. 1993. Evidence for a *Wolbachia* symbiont in *Drosophila melanogaster*. *Genet Res*. 62: 23-29.

Horsfall, W. R., Fowler, H. W., Moretti, L. J. and Larsen, J. R. 1973. *Bionomics and Embryology of the Inland Floodwater Mosquito Aedes vexans*. University of Illinois Press, Urbana, 211 pp.

Hoshizaki, S. and Shimada, T. 1995. PCR-based detection of *Wolbachia*, cytoplasmic incompatibility microorganisms, infected in natural populations of *Laodelphax striatellus* (Homoptera: Delphacidae) in Central Japan: Has the distribution of *Wolbachia* spread recently? *Insect Mol. Biol.* 4(4): 237-243.

Humeres, S. G., Gardenal, C. N., Almiron, W., Sereno, R. and Sabattini, M. S. 1990. *Culex* species (Diptera: Culicidae) from central Argentina: identification by electrophoretic zymograms and genetic relationships. *J Med Entomol.* 27(5): 784-788.

Hurst Gregory, D., Jiggins Francis, M., Schulenburg, J., Bertrand, D., West Stuart, A., Goriacheva, I., Zakharov, I. A., Werren, J. H., Stouthamer, R. and Majerus, M. 2000. Male-killing *Wolbachia* in two species of insect. *Proceedings of the Royal Society Biological Sciences*. Series B. 266 (1420): 735-740.

Ibarra, J. E. and Federici, B. A. 1986b. Parasporal bodies of *Bacillus thuringiensis* subsp. *morrisoni* (PG-14) and *Bacillus thuringiensis*, subsp. *israelensis*, are similar in protein composition and toxicity. *FEMS Microbiol. Lett.* 34(1): 79-84.

Igbokwe, E. C. 1988. Heterosis associates with DOPA-oxidase dimorphism in *Culex pipiens. Comparative Biochemical Physiology* 89B: 5-7.

Igbokwe, E. C. and Braden, P. 1989. Genetics of alkaline phosphatase variability in *Culex quinquefasciatus* (Diptera: Culicidae). *American Zoology* 29(4): 7A.

Jammongluk, W., Kittayapong, P., Baisley, K. and O'Neill Scott, L. 2000. *Wolbachia* infection and expression of cytoplasmic incompatibility in *Armigeres subalbatus* (Diptera: Culicidae). *J. Med. Entom.* 37(1): 53-57.

Jiggins, F. M., Bently, J. K., Majerus, M. E. and Hurst, G. D. 2001. How many species are infected with *Wolbachia*? Cryptic sex ratio distorters revealed to be common by intensive sampling. *Proceedings of the Royal Society of London*- Series B: Biological Sciences. 268(1472): 1123-1126.

Karabatsos, N. 1985. International catalogue of arboviruses: including certain other viruses of vertebrates, 3rd edition. *American Society of Tropical Medicine and Hygiene* 1, 147 pp.

Kitzmiller, J. B. 1950. Fertility in species crosses in mosquitoes. *Entomol. News* 61: 130-131.

Kettle, D. S. 1995. *Medical and Veterinary Entomology*. CAB International, 2nd ed., Oxon, UK, 725 pp.

Laven, H. 1967. Formal Genetics of *Culex pipiens*. *In Genetics of Insect Vectors of Disease.* Elsevier Publishing Co., Amsterdam. 17-65pp.

Lehane, M. J. 1991. *Biology of blood-sucking insects*. Harper Collins Academic, London, UK, 288 pp.

Linnaeus, C. 1758. *System. Naturae* 10: 602.

Lessios, H. A. 1992. Testing electrophoretic data for agreement with Hardy-Weinberg expectations. *Mar. Biol*. 112:517-523.

Levene, H. 1949. On a matching problem arising in genetics. *Ann. Math. Statist*. 20: 91-94.

MacDonald, J. F. and Ayala, F. J. 1974. Genetic response to environmental heterogeneity. *Nature* 250: 572-574.

Margalit, J. and Dean, D. 1985. The story of *Bacillus thuringiensis israelensis* (B.t.i). *J. Am. Mosq. Ass*. 1: 1-7.

Markert, C. L. and Moller, F. 1959. Multiple forms of enzymes: tissue, ontogenic, and species specific patterns. *Pro. Nat. Acad. Sci*. 45: 753-763.

Monath, T. P. 1998. The arboviruses. *Epidemiology and ecology*. CRC Press, Boca Raton, FL. Vls. 1-5.

Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martelli, G. P., Mayo, M. A. and Summers, M. D. 1995. *Virus Taxonomy–Classification and Nomenclature of Viruses*. Sixth Report of the International Committee on Taxonomy of Viruses. Springer-Verlag, Wien, New York. 586 pp.

Murphy, R. W. et al. 1996. Proteins: isozyme electrophoresis. In Hillis, D.M., Moritz, C. and Mable, B.K. (eds.): *Molecular Systematics*. Sinauer Associates, Inc. Publishers Sunderland, Massachusetts U.S.A. pp. 51-120.

Narang, S., Bhalla S. C. and Narang, N. 1977. Isozymes of *Culex p. fatigans*. I. An esterase locus in linkage Group III and its variability in natural populations. *Journal Hereditary* 68: 96-99.

Nei, M. 1972. Genetic distance between populations. *Am. Natur*. 106: 283-292.

Nei, M. and Saitou, N. 1987. *Molecular Evolutionary Genetics*. Columbia Uni. Press, New York.

Norkrans, B. 1980. Surface microlayers in aquatic environments. *Advances in Microbial Ecology*. 4: 51-85.

O'Neill, S., Giordano, R., Colbert, A. M. E., Karr, T. L and Robertson, H. M. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl. Acad. Sci.* USA 89: 2699-2702.

O'Neill, S. L. 1999. *Wolbachia*: why these bacteria are important to genome research. *Microb Comp Genomics.* 4: 159.

Oopijnen Tbreeuwer, J. A. 1999. High temperatures eliminate *Wolbachia*, a cytoplasmic incompatibility inducing endosymbiont, from the two-spotted spider mite. *Exp Appl Acarol* 23(11): 871-881.

Palade, G. E. and Siekevitz, P. 1956. Liver microsomes. An integrated morphological and biochemical study. *J. Biophysic. and Biochem. Cytol*. 2: 171.

Pasteur, N., Sinere, G. and Gabinaud, A. 1981. Esterase -2 and Esterase-3 polymorphism in *Culex pipiens* L. from Southern France in relation to organophosphate resistance. *Biochemical Genetics* 19: 499-517.

Pryor, S. C., Cheng, M., Ferrell, R. E. and Hacker, C. S. 1979. Biochemical genetics of the Culex Pipiens Complex, I 6-phosphogluconate dehydrogenase. The University of Texas Health Science Center, Houston, Texas, USA.

Pryor, S. C. and Ferrell, R. E. 1981. Biochemical genetics of the Culex Pipiens Complex-III. Alpha-lycerophosphate dehydrogenase. *Comparative Biochemical Physiology* 69B:3-28.

Pryor, S.C. and Daly, J. 1991. Temporal variation in morphological and genetic characteristics within a hybrid populations of *Culex pipiens* (Diptera: Culicidae). *J. Med. Entomol*. 28: 481-486.

Puri, B., Henchal, E. A. and Burans, J. 1994. A rapid method for detection and identification of flaviviruses by polymerase chain reaction and nucleic acid hybridization. *Arch. Virol*. 134: 29-37.

Rasgon J. and Scott, T. 1999. Evaluation of a dot-blot assay for detecting *Wolbachia* infection in mosquitoes. *Mosquito control annual research*. University of California Press. 81-84 pp.

Read, K. and Ellis-Pegler, R.B. 2001. Dengue fever in returned travellers. *NZ Med J.* 114(1130): 194.

Rigaud, T., Antoine, D., Marcade, I. and Juchault, P. 1997. The effect of temperature on sex ratio in the isopod *Porcellionides pruinosus*: Environmental sex determination or a by-product of cytoplasmic sex determination? *Evol. Ecol*. 11(2): 205-215.

Rokas, A., Atkinson, R. J., Brown, G. S., West, S. A. and Stone, G. N. 2001. Understanding patterns of genetic diversity in the oak gallwasp *Biorhiza pallida*: demographic history or a *Wolbachia* selective sweep? *Heredity*. 87(Pt 3): 294-304.

Roth, T. F. and Porter, K. R. 1962. *Specialized sites on the cell surface for protein uptake in 5th International Congress for Electron Microscopy*, Philadelphia, (S.S. Breese, Jr., editor), New York, Academic Press, Inc., 2, LL-4.

Roth, T. F. and Porter, K. R. 1963. Membrane differentiation for protein uptake. *Fed. Proc.* 22: 2.

Roth, T. F. and Porter, K. R. 1964. Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti* L. *Journal of Cell Biology*. 20: 313-330.

Rousset, F., Vautrin, D. and Silignac, M. 1992. Molecular identification of *Wolbachia*, the agent of cytoplasmic incompatibility in *Drosophila simulans*, and variability in relation with host mitochondrial types. *Proc R Soc Lond B Biol Sci.* 247: 163-168.

Sang, C. T., Cuzzubo, A. J. and Devine, P. 1997. Evaluation of a Commercial Capture Enzyme-Linked Immunosorbent Assay for Detection of Immunosorbent Assay for Detection of Immunoglobulin M and G Antibodies Produced during Dengue Infection. *American Soc. for Micro*. USA. 5: 7-10.

Schutz, Steven J. and Eldrige, B. 1993. *Mosquito Systematics*. 25(3): 170-176.

Scott, F. 1980. *Biology.* Scott, Foresman and Company, Glenview, Illinois. 689 pp.

Shaw, C. R. and Prasad, R. 1970. Starch gel electrophoresis of enzymes-a compilation of recipes. *Biochem. Genet.* 4: 297-320.

Simon J. P. 1969. Esterase isozymes in the mosquito, *Culex pipiens fatigans:* development and genetic variation. *Annual Entomological Society of America*. 62: 1307-1311.

Singh, K. R. P. and Brown, A. W. A. 1957. Nutritional requirements of *Aedes aegypti* L. *J. Insect Physiol*. 1: 109.

Sironi, M., Bandi, C., Sacchi, L., Di Sacco, B., Damiani, G. and Genchi, C. 1995. Molecular evidence for a close relative of the arthropod endosymbiont *Wolbachia* in a filarial worm. *Mol Biochem Parasitol*. 74: 223-227.

Smith, A. H. 1959. Follicular permeability and yolk formation. *Poultry Sc*. 38, 1437pp.

Smith, D. 1968. *Insect cells: their structure and function*. Oliver and Boyd, Edinburgh, Great Britain. 337-340.

Smith, C. A. B. 1970. A note on testing the Hardy-Weinberg law. *Ann. Hum. Genet*. 33: 377-383.

Spiess, E. B. 1989. *Genes in populations*. John Wiley & Sons, New York.

Steiner, W. W. M. and Joslyn, D. J. 1979. *Electrophoretic techniques for the genetic study of mosquitoes. Department of Genetics and Development*, University of Illinois, Urbana, Illinois.

Stouthamer R., Breeuwer, J. A. and Hurst, G. D. 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annu Rev Microbiol*. 53: 71-102.

Sumarmo, S. and Suroso, T. 1987. Economic impact of dengue haemorrhagic fever in Jakarta, Indonesia. *Dengue Newsletter*. Vol. 13.

Sundararaman, S. 1949. Biometrical studies on intergradation in the genitalia of certain populations of *Culex pipiens* and *Culex quinquefasciatus* in the United States. *Am. J. Hyg.* 50: 307-314.

Tabachnick, W. and Powell, J. 1983. Genetic analysis of *Culex pipiens* populations in the Central Valley of California. *Ann. Entomol. Soc. Am*. 76: 715-720.

Tai Min, K. and Benzer, S. 1997. *Wolbachia*, normally a symbiont of *Drosophila*, can be a virulent, causing degeneration and early death. *Proc. Natl. Acad. Sci*. USA. 94: 10792-10796.

Taylor, M. J. and Hoerauf, A. 1999. *Wolbachia* bacteria of filarial nematodes. *Parasitology Today* 15: 437-442.

Telford, A. D. 1963. A consideration of diapause in *Aedes nigromaculis* and other aedine mosquitoes (Diptera: Culicidae). *Ann. Ent. Soc. Am*. 56(4): 409-418.

Tibayrene, M. 1979. Isozymes and medical entomology. *Cah. Orstom Ser. Ent. Med. et Parasitology*. 17(4): 249-256.

Urbanelli, S. and Bullini, L. 1985. Electrophoretic studies on *Culex quinquefasciatus* Say from Africa: genetic variability and divergence from *Culex pipiens* L. (Diptera: Culicidae). *Bulletin of Entomological Research*. 75: 291-304.

Urbanelli, S., Sivestrini, F., Sabatinelli, G., Raveloarifera, F., Petrarca, V. and Bullini, L. 1995. Characterization of the Culex Pipiens Complex (Diptera: Culicidae) in Madagascar. *J. Med. Entomol*. 32(6): 778-786.

Vithayasai, C. 1973. Exact critical values of the Hardy-Weinberg test statistic for two alleles. *Communs. Statist*. 1: 229-242.

Wagner, R. P. and Selander, R. K. 1970. Isozymes in insects and their significance. *Ann. Rev. Entomology*. 19: 117-138.

Weir, B. S. 1990. *Genetic data analysis*. Sinauer Associates, Inc. Publ. Sunderland, Massachusetts.

Werren, J. H., Zhang, W. and Guo, L. R. 1995. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. *Roc R Soc Lond B Biol Sci.* 261: 55-63.

Werren, J. H. 1997. Biology of *Wolbachia*. *Ann. Review of Entomology* 42: 587-609.

Werren, J. H. and Bartos, J. D. 2001. Recombination in *Wolbachia*. *Current Biology*. 11(6): 431-435.

Wernsdorfer, W. H. 1980. The importance of malaria in the world. In: *Malaria*, Volume 1 (J. P. Kreier, ed.) Academic Press, New York, 1-93pp.

WHO, 1993. Tropical disease Research: Progress 1991-92, 11th programme report of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), World Health Organization, Geneva, 134 pp.

WHO, 1997a. World malaria situation in 1994. Part I. Population at risk. *Weekly Epidem. Rec.* 72(36): 269-274.

WHO, 1997b. World malaria situation in 1994. Part III. Europe, South-East Asia, Western Pacific. *Wkly. Epidem. Rec*. 72(38): 285-290.

Wright, J. D., Sjöstrand, F. S., Portaro, L. K. and Barr, A. R. 1978. The ultrastructure of the rickettsia-like microorganisms *Wolbachia pipientis* and associated virus like bodies in the mosquito *Culex pipiens. J Ultrastruc Res.*, 63: 79-85.

Wright, J. and Barr, A. 1980. Ultrastructure and symbiotic relationships of *Wolbachia* of mosquitoes of the *Aedes scutellaris* group. *J. Ultrastruc. Res*. 72: 52-64.

Wright, J. D. and Barr, A. R. 1981. *Wolbachia* and the normal and incompatible eggs of *Aedes polynesiensis* (Diptera: Culicidae*). J. Invert. Pathol* 38: 409-418.

Yen, J. H. and Barr, A. R. 1971. *Nature* 232: 657-658.

Yen, J. H and Barr, A. R. 1974. Incompatibility in *Culex pipiens*. *The Use of Genetics in Insect Control.* Elsevier/North Holland, Amsterdam, 97-118.

Yen, Y.H. 1975. Transovarial transmission of rickettsia-like microorganisms in mosquitoes. *Ann. NY Acad. Sci*. 266: 152-161.

Yoon, S. 1987. Community participation in the control and prevention of DF/DHF: Is it possible? *Dengue Newsletter*. Vol. 13.

Zimmer, C. 2001. *Wolbachia*: A tale of Sex and Survival. *Science*. 292: 1093-1095.

9. APPENDIX

Appendix 2-1. Chi- square adaption test after Hardy-Weinberg in population of *Culex p. pipiens* (see 2.2.10).

Locus	Genotype	Frequency		X^2-values or number of heterozygotes
		Observed	Expected	
MDH $_{NAD}$-2*	100/124	1	1.9487	
	100/100	10	18.0256	1-3 heterozygotes
IDH-2*	84/84	1	0.2877	
	84/100	5	6.4250	
	100/100	31	30.2873	4-8 heterozygotes
HK-1(2,3)*	100/100	5	6.6327	
	100/117	16	12.7347	
	117/117	4	5.6327	1.3417
MPI*	31/131	1	0.0978	
	76/76	1	0.2936	
	76/124	1	1.5283	
	76/162	1	0.7075	
	76/177	2	0.2354	
	100/100	4	1.0780	
	100/114	1	0.2153	
	100/124	1	2.8034	
	100/162	1	1.2941	
	124/124	3	1.5294	
	124/145	1	1.0193	
	124/162	4	1.5296	
	131/131	2	0.1958	
	138/190	1	0.0392	
	145/145	1	0.1176	
	145/190	1	0.1570	
Alleles MPI*31,76,100,114,131,138,145,162,177 & 190 were combined (31*+.) & the new frequencies were computed				
	31*+../31*+..	16	14.5294	
	31*+../124*	7	9.9412	
	124/124	3	1.5294	1.8051

Appendix 2-2. Chi- square adaption test after Hardy-Weinberg in population of *Culex p. quinquefasciatus* (Phils.).

Locus	Genotype	Frequency		X^2-values or number of heterozygotes
		Observed	Expected	
IDH-2*	72/72	1	0.1586	
	84/84	1	0.01595	
	72/100	3	4.5222	
	100/100	27	25.33119	
	Alleles IDH*72 & 84 were combined & the new frequencies were computed			
	100/84+72	3	6.3346	
	72+84/72/84	2	0.3335	4-8 heterozygotes
MPI*	76/76	1	0.5712	
	76/100	5	4.9993	
	76/124	2	2.5705	
	100/100	10	9.4453	
	100/114	1	0.5547	
	100/124	8	9.9987	
	100/162	1	0.5547	
	124/124	4	2.4277	
	Alleles MPI*76,114,124,& 162 were combined & the new frequencies were computed			
	76*+../76*..	7	6.4437	
	76*+../100	15	16.1110	0.1033
PGM*	58/100	3	3.5702	
	58/150	2	1.0313	
	100/100	17	15.7132	
	100/150	7	9.2842	
	100/183	1	0.7131	
	150/150	2	1.2378	
	Alleles PGM*58,150 & 183 were combine and the new frequencies were computed			
	58+../58+..	4	2.7148	
	58+../100	11	13.5702	0.8598

Appendix 2-3. Chi-square adaption test after Hardy-Weinberg in population of *Culex p. quinquefasciatus* (USA). The genotype frequencies of GPI* showed significant deviations from Hardy- Weinberg equilibrium.

Locus	Genotype	Frequency		X^2-value or number of heterozygotes
		Observed	Expected	
MPI*	76/100	3	2.4518	
	76/138	1	0.2581	
	100/100	4	5.5171	
	100/138	1	1.2259	
	100/124	3	1.8398	
	100/145	4	2.4518	
	Alleles MPI*76,124,138 & 145 were combined and the new frequencies were computed			
	76*+../76*+..	1	2.5155	
	76*+../100	11	7.9674	1.8994
GPI*	75/100	16	10.1417	
	100/100	5	7.9256	**4.0047**

Appendix 3-1. Preparation of 1% OsO_4

chemical	quantity
OsO_4 (4%)	1 mL
aqua bidest	1 mL
Kaliumhexacyanoferrat-II	2 mL

Appendix 3-2. Preparation of 1% uranyl acetate in 0.05 M malic buffer (pH 5.2)

chemical	quantity
uranyl acetate	0.1 g
malic buffer	10 ml

Appendix 3-3. Preparation of Spurr pur medium

chemical	quantity
vinylcyclohexenedioxide or VCD	10 g
diglycidyl ether of propylene glycol	6 g
nonenyl succinic acid anhydride (NSA)	26 g
dimethyl-aminoethanol	0.4 g

Appendix 3-4. Preparation of Spurr pur and absolute alcohol with 1:1 ratio

chemical	quantity
Spurr medium	6 mL
absolute alcohol	6 mL

Appendix 3-5. Preparation of 3:1 ratio of Spurr pur and absolute alcohol

chemical	quantity
absolute ethanol	3 mL
Spurr pur	9 mL

Appendix 5-1. Blood serum samples in plate 1 with PANBIO units from 1 to 10.

Sample	**Absorption by 450nm**	**PANBIO units**	**Result**
female	0.375	9.54	EV (=)
female	0.356	9.06	EV (=)
female	0.182	4.63	-
female	0.092	2.34	-
female	0.188	4.78	-
female	0.426	10.84	EV (=)

Legend: PANBIO units <9 = negative; 9-11 = equivocal ; >11 = positive
Absorption cut off= 0.393

Appendix 5-2. Blood serum samples in plate 1 with PANBIO units from 11 to 20.

Sample	Absorption by 450 nm	PANBIO units	Result	Sample	Absorption by 450 nm	PANBIO units	Result
female	0.733	18.65	+	female	0.754	19.19	+
female	0.751	19.11	+	female	0.760	19.34	+
female	0.823	20.94	+	female	0.795	20.23	+
female	0.751	19.11	+	female	0.792	20.15	+
female	0.711	18.09	+	female	0.727	18.50	+
female	0.805	20.48	+	female	0.628	15.98	+
female	0.667	16.97	+	female	0.686	17.46	+
female	0.651	16.57	+	female	0.824	20.97	+
female	0.552	14.05	+	female	0.630	16.03	+
female	0.819	20.84	+	female	0.550	14.00	+
female	0.775	19.72	+	female	0.748	19.03	+
female	0.762	19.39	+	female	0.718	18.27	+
female	0.747	19.01	+	female	0.811	20.64	+
female	0.758	19.29	+	female	0.751	19.11	+
female	0.817	20.79	+	female	0.790	20.10	+
female	0.641	16.31	+	female	0.665	16.92	+
female	0.742	18.88	+	female	0.703	17.89	+
female	0.730	18.58	+	female	0.595	15.14	+
female	0.596	15.17	+	female	0.906	23.05	+
female	0.822	20.92	+	female	0.555	14.12	+
female	0.582	14.81	+				

Legend: PANBIO units<9 = negative; 9-11 = equivocal ; >11= positive
Absorption cut-off= 0.393

Appendix 5-3. Blood serum samples in plate 1 with PANBIO units from 21 to 30.

Sample	Absorption by 450 nm	PANBIO units	Result	Sample	Absorption by 450 nm	PANBIO unit	Result
female	0.849	25.45	+	female	0.986	25.09	+
female	0.939	23.89	+	female	0.924	23.51	+
female	0.850	21.63	+	female	1.027	26.13	+
female	0.956	24.33	+	female	0.852	21.70	+
female	0.934	23.80	+	female	0.869	22.11	+
female	0.846	21.53	+	female	0.836	21.27	+
female	0.840	21.37	+	female	0.971	24.71	+
female	0.913	23.23	+	female	0.871	22.16	+
female	0.896	22.80	+	female	0.873	22.21	+
female	0.973	24.76	+	female	0.965	24.55	+
female	0.980	24.94	+	female	0.842	21.42	+
female	0.997	25.37	+	female	0.866	22.04	+
female	0.876	22.29	+	female	0.945	24.05	+
female	0.987	25.11	+	female	0.922	25.46	+
female	0.920	23.41	+	female	0.902	22.95	+
female	0.906	23.05	+	female	0.961	24.45	+
female	0.848	21.58	+	female	0.950	24.17	+
female	0.934	23.77	+	female	0.841	21.40	+
female	0.957	24.35	+	female	1.001	25.47	+

female	0.865	22.01	+	female	0.844	21.48	+
female	0.855	21.75	+	female			

Legend: PANBIO units<9= negative ; 9-11= equivocal; >11= positive
Absorption cut-off= 0.393

Appendix 5-4. Blood serum samples in plate 2 with PANBIO units from 1 to 10.

Sample	Absorption by 450 nm	PANBIO units	Result
female	0.292	9.15	EV
male	0.349	10.94	EV
male	0.185	5.80	-

Legend: PANBIO units<9= negative ; 9-11= equivocal ; >11= positive
Absorption cut-off= 0.319

Appendix 5-5. Blood serum samples in plate 2 with PANBIO units from 11 to 20.

Sample	Absorption by 450nm	PANBIO units	Result	Sample	Absorption by 450nm	PANBIO units	Result
female	0.545	17.08	+	female	0.602	18.87	+
female	0.425	13.32	+	male	0.603	18.90	+
male	0.611	19.15	+	female	0.449	14.08	+
male	0.649	20.34	+	female	0.650	20.38	+
male	0.656	20.60	+	male	0.418	13.10	+
male	0.464	14.55	+	male	0.542	16.99	+
male	0.505	15.83	+	male	0.586	18.37	+
male	0.660	20.69	+	male	0.409	12.82	+
male	0.571	17.90	+	male	0.517	16.21	+
male	0.566	17.74	+	male	0.560	17.55	+
male	0.373	11.70	+	male	0.354	11.10	+

Legend: PANBIO units<9= negative; 9-11= equivocal; >11= positive
Absorption cut-off= 0.319

Appendix 5-6. Blood serum samples in plate 2 with PANBIO units from 21 to 30.

Sample	Absorption by 450 nm	PANBIO units	Result	Sample	Absorption by 450 nm	PANBIO units	Result
female	0.719	22.54	+	female	0.872	27.34	+
female	0.832	26.08	+	female	0.752	23.57	+
female	0.836	26.21	+	female	0.955	29.94	+
female	0.861	26.99	+	female	0.686	21.50	+
male	0.786	24.64	+	male	0.819	25.67	+
male	0.734	23.01	+	male	0.849	26.61	+
male	0.902	28.27	+	male	0.785	24.61	+
male	0.793	24.86	+	male	0.937	29.37	+
male	0.948	29.72	+	male	0.931	29.18	+
male	0.824	25.83	+	male	0.711	22.29	+

male	0.833	26.11	+	male	0.733	22.98	+
male	0.862	27.02	+	male	0.734	23.01	+
male	0.913	28.62	+	male	0.954	29.91	+
male	0.916	28.71	+	male	0.844	26.46	+
male	0.843	26.43	+	male	0.807	25.30	+
male	0.696	21.82	+	male	0.853	26.74	+
male	0.687	21.54	+	male	0.950	29.78	+
male	0.682	21.38	+	male	0.673	21.09	+
male	0.675	21.16	+	male	0.906	28.40	+
male	0.775	24.29	+	male	0.692	21.70	+
male	0.807	25.30	+	male	0.897	28.12	+
male	0.786	24.64	+	male	0.684	21.44	+
male	0.817	25.61	+	male	0.671	21.03	+
male	0.687	21.54	+	male	0.870	27.27	+

Legend: PANBIO units<9= negative ; 9-11= equivocal ; >11= positive
Absorption cut-off= 0.319

Appendix 5-7. Blood serum samples in plate 2 with PANBIO units from 31 to 40.

Sample	**Absorption by 450nm**	**PANBIO units**	**Result**	**Sample**	**Absorption by 450nm**	**PANBIO units**	**Result**
female	1.044	32.73	+	female	1.041	32.63	+
female	1.087	34.08	+	female	1.010	31.66	+
female	1.135	35.58	+	female	1.115	34.95	+
male	1.097	34.39	+	male	1.037	32.51	+
male	0.090	31.35	+	male	1.038	32.54	+
male	1.041	32.63	+	male	1.043	32.70	+

Legend: PANBIO units<9= negative ; 9-11= equivocal ; >11= positive
Absorption cut-off= 0.319

Appendix 6-1. Occupations of the respondents.

Occupations	Percentage
housewives	21.56%
vendors	18.87%
private employees	16.71%
artisans	13.48%

Appendix 6-2. Educational attainment of the respondents.

Educational attainment	Percentage
college	36.66%
high school	40.97%
elementary	14.02

Appendix 6-3. Location of the houses that were surveyed.

Location of houses	Percentage
near to the open canals	49.06%
near the river	12.13%
along the road	19.68
low elevation area	9.43%
near partially close canals	8.09%
with several plants	6.2%
beside the market	1.62%

Appendix 6-4. House decription of the respondents.

House description	Percentage
combination of cement and wood	57.41%
plain wood	26.95%
concrete	11.59
unspecified	4.04%
water sealed comfort room	74.12%
open pit comfort room	20.22%
unknown comfort room	5.66%
with electric power	98.04%
have access to the main road	25.14%
have telephone	33.24%

Appendix 6-5. Sources of water among the households.

Sources of water	Percentage
direct water	64.42%
collect water from a communal faucet	26.95%
uses rain water	5.39%
get water from well	3.23%
get water from artisan well	2.96%

Appendix 6-6. Percentage of people's knowledge on the symptoms of dengue fever.

Symptom	% of people's knowledge on the symptom
fever	64.96%
nose bleeding	32.08%
rash	25.43%
headache	17.79%
nausea & vomiting	16.7%
waekness	11.86%
body aches	9.43%
retro-orbital pain	5.12%

Appendix 6-7. Peoples's complains on fogging.

Different complaints on fogging	Percentage of the respondents
complain of respiratory symptoms	12.94%
complain that the mosquitoes return	12.94%
complain of neurologic symptoms	3.23%
complain the noise of the fogging machine	0.54%

Appendix 6-8. Vector control measures at the household level that were reported by the respondents.

Vector control measures	Pecentage of the respondents
cleaning the surroundings	52.83%
mosquito coil	31.27%
mosquito nets	30.19%
spray	28.84%
electric fan	24.80%
screen the house	9.16%
burning of plant matter	8.89%
cover the water containers	7.82%
cleaning of gutters	4.31%
change water in the flower vase	3.77%