Characterization and expression profiles of Glutathione - S- transferase (GSTs) gene in *Anopheles* mosquito vector complex

Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in Department of Zoology

by

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CERTIFICATE

I certify that the thesis entitled '**Characterization and expression profiles of Glutathione - S-transferase (GSTs) gene in Anopheles mosquito vector complex**' submitted to the Mizoram University for the award of a degree of Doctor of Philosophy in Zoology Department by **Mr. K. VANLALHRUAIA** is a record of research work carried out by him during the period from 2009 to 2013 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles in this University or any other University or institution of higher learning.

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I. INTRODUCTION AND REVIEW OF LITERATURE

II. INTRODUCTION AND REVIEW OF LITERATURE

2.1 International and national status of mosquito species

Mosquitoes (Diptera: Culicidae) and mosquito-borne diseases have been threatening human and animals. There are 38 genera of mosquitoes worldwide wherein three genera (*Anopheles, Aedes* and *Culex*) were the most important one transmitting dengue fever, yellow fever, malaria, filariasis, chikungunya and encephalitis (Adityaa *et al*., 2006). No part of the world is free from vector borne diseases. Mosquito-borne parasitic diseases are endemic in many areas of the world, causing more than 3.2 billion people to be at risk (WHO, 1998). There are 444 formally named species and 40 unnamed members of species complexes recognized as distinct morphological and/or genetic species of *Anopheles* in the world (Harbach, 2004). In India, 58 species has been described, six of which have been implicated to be main malaria vectors, namely *Anopheles culicifacies*, *Anopheles dirus*, *Anopheles fluviatilis*, *Anopheles minimus*, *Anopheles stephensi* , and *Anopheles sundaicus*. Of the 58 species of *Anopheles* found in India, *Anopheles dirus, Anopheles fluviatilis* and *Anopheles minimus* are primary vectors of malaria in NE India (Nagpal and Sharma, 1987). Each year 300 to 500 million cases of malaria are reported worldwide, resulting in 1.5 to 2.7 million deaths (Centers for Disease Control and Prevention, 2004). India is on 18th position in the total reported malaria cases and on 21st position in reported malaria deaths (NVBDCP, 2013). Among 111 *Aedes* species prevalence in India, *Aedes aegypti* and *Aedes albopictus* were responsible for 765 deaths (2007-2012) due to dengue fever. Moreover, there were numbers of deaths due to Japanese encephalitis (181 deaths in 2011). Thus, vector borne diseases were one of the diseases that killed numbers of people in India till today.

1.2. Status of Malaria and Mosquito management in Mizoram

Mizoram (92.15-93.29^{o} E and 21.58-24.35^{o} N) is one of the Seven Sister States (Malaria endemic area) listed as in North Eastern India, sharing borders with the states of Tripura, Assam, Manipur and with the neighboring countries of Bangladesh and Burma. It belongs to the Indo-Burma region, which is one of the biodiversity hotspot identified in the world by Myers *et al.* (2000). Mizoram has an average rainfall of 2094.85 mm p.a., average relative humidity of 77% (within 2007-2009) and a moderate climate (Economics & Statistics dept. Govt. of Mizoram, 2010). Mizoram is a land of hilly area wherein more than 70% of the total population is engaged in some forms of agriculture (Jhum or shifting cultivation), most of the malaria positive cases are still contributed by agricultural field's workers (Health Dept. Govt. of Mizoram, 2010).

In India, Mizoram alone contributed 5.73% of deaths due to malaria in 2007 and 10.44% in 2010 (NVBDCP, 2013). The record of the Mizoram State Vector Borne Diseases Control Programme (MSVBDCP) stated that in 2011, malaria was highest in Lunglei district (40.42% of total positive cases) followed by Lawngtlai district (29.88%) and the least was Champhai district (0.89%). Combat against malaria started since 1957 as the name 'National Malaria Control Programme' (NMCP) which was Government of India flagship programme. In 1958, NMCP was changed to National Malaria Eradication Programme that reflected the action in Mizoram that IRS (Indoor Residual Spray) of organochlorine insecticide, DDT (1 kg of DDT 50% effective conc. dissolved in 10 L of water *i.e.* 5% conc.) was started since 1960s till today (Health dept. Govt. of Mizoram, 2012). Moreover, toward vector control and management of malarial cases, the Directorate of NMCP was renamed as Directorate of NVBDCP in 2003 which was

financially assisted by GFATM (Global Fund for AIDS, Tuberculosis and Malaria) and World Bank project, under which MSVBDCP (Mizoram State Vector Borne Diseases Control Programme) has started distribution of 1% K-othrine, a synthetic pyrethroid (Deltamethrine 2.5% active ingredient v/v) for treated bed-nets all over Mizoram which was replaced by distributions of Long lasting insecticidal nets (Olyset net *ie.*Permethrine incorporated into polyethylene) since 2008 (Health Dept. Govt. of Mizoram, 2012). MSVBDCP (Mizoram State Vector Borne Diseases Control Programme) has also been setting up ASHA (Accredited Social Health Activist) and MPW (Malaria Programme Worker) in all towns and remote villages to take blood slides of fevers as earliest possible and to give treatment on time. Malaria being the main cause of death in Mizoram and 119 persons died of malaria with 79 males and 40 females during the year 2009 (NVBDCP-Mizoram report, 2010).

1.3. Distribution, abundance and breeding habitats

Mosquito species abundance and habitats in the Himalayan region had been extensively studied (Devi and Jauhari, 2007; Bhatt, 1975). Thu survival of immature and emergence of pupae into adults had a good correlation to the altitude and temperature of the breeding habitats (Devi and Jauhari, 2007). The prevalences of *Culex mimeticus, Anopheles maculatus, Aedes albopictus, Aedes albolateralis, Anopheles fluviatilis, Anopheles splendidus, Anopheles nigerrimus, Anopheles culicifacies, Anopheles stephensi, Anopheles vagus*, *Culex vishnui*, *Aedes thomsoni, Culex brevipalpis, Culex quinquefasciatus, Anopheles lindesayi, Anopheles gigas, Aedes subalbopictus, Culex viridiventer, Culex barraudi, Culex vagans* and *Aedes dissimilis* has been documented by Devi and Jauhari (2007) but no reported of *Culiseta* spp. In Northeastern region of India, mosquitoes's surveys in various states have been carried out to study the

occurrence, distributions, species composition and identification of vector (Sen *et al.*, 1973; Rajagopal, 1979; Malhotra *et al.,* 1982, 1984; Sarkar *et al.*, 1984). In 1987, mosquito faunal survey carried out by Nagpal and Sharma in Northeastern region of India reported the prevalences of 60 species of mosquitoes, in which the most dominant genus was *Anopheles* followed by *Culex, Aedes* and *Mansonia*.

There was a few information regarding Mosquito prevalence, geographical distribution and relative density of potential vector populations described from Mizoram. The first Anopheline survey was done by Das and Baruah (1985) that indicated *Anopheles dirus* and *Anopheles minimus* as malarial vector. Later, in 1987 the survey carried out by Nagpal and Sharma reported the presence of *Anopheles barbirostris, Anopheles nigerrimus, Anopheles aconitus, Anopheles annularis, Anopheles karwari, Anopheles kochi, Anopheles maculates, An, majidi, Anopheles nivipes, Anopheles philipinensis, Anopheles theobaldi, Anopheles vagus, Anopheles willmori* and *Anopheles kuchingensis*, but no record of *Anopheles dirus* and *Anopheles minimus.* Das *et al*. (1990) reported the presence of *Anopheles dirus* and *Anopheles minimus* from Tlabung, south of Mizoram that has been incriminated as a vector of malaria. Apart from this, mosquito faunal survey carried out by Malhotra (1994) stated the prevalences of *Aedes albolateralis, Aedes chrysolinea, Aedes novoniveus barraud, Aedes poecilus, Aedes albopictus, Culex bitaenlorhynchus, Culex gelidus, Culex mimeticus, Culex quinquefasciatus, Culex sinensis, Culex tritaeniorhynchus, Culex vishnui* and *Culex fuscanus* in Mizoram, but no reported of *Culiseta* species.

Understanding where mosquitoes breed and why they prefer certain water bodies over others is vital for designing mosquito control strategies. Knowing the ecology and behavior of a vector is essential to determine its role in disease transmission and the type of control measures

that may be appropriate for it (Shililu *et al.*, 2003). Several work being done through distribution and types of larval breeding habitats. Bates (1947) classified 7 different types of major larval habitats depending on size, degree of persistance of the breeding water (permanent and temporary) and types of water (WHO, 1975). Mosquitoes are more abundant in temporary breeding places while others occurred usually in permanent ones (Almiron and Brewer, 1994), The resources in terms of food, predators and competitors present in the habitat determine the population status of larval mosquitoes, both qualitatively and quantitatively (Adityaa *et al*., 2006). A number of studies have been carried out on mosquito breeding in various habitats (Iyengar, 1932; Russell and Rao, 1972; Rahman *et al*., 1973; Rajagopalan *et al*., 1979; Sahu *et al*., 1990; Bhatt *et al*., 1993), one of the most important factor was the vegetation that favours larval propagation and is correlated with adult densities (Savage *et al*., 1990; Rajmankova *et al*., 1992; Rodriguez *et al*., 1993; Rajnikant, 1996). Small and open habitats are more productive and selected for oviposition sites compared to large larval habitats during the rainy seasons (Mwangangia *et al*., 2007). Adult mosquitoes are found in habitats like human dwelling, cattle shed, mixed dwelling and other outdoor resting sites such as bushes, tree holes, rock holes and discarded containers such as used tyres and plastic receptacles (Devi and Jauhari, 2008; Dabire *et al*., 2008).

1.4. Environmental factors

The distribution and abundance of an insect species depends on its own biological characteristics and the influence of other organisms, on its physical environment. Weather plays a major role (Bayoh *et al*., 2003; Reisen *et al*., 2006), as insects are poikilothermic or cold-blooded. Metabolic heat, which is generated by most insects themselves, is limited and has little effect on their body temperature. Therefore, their metabolic rate and the growth and development

rate of insects depend on the temperature of their direct environment. Temperature, rainfall and relative humidity are physical factors that influence the abundance of the mosquitoes (Lee, 1990). If the water temperature rises, the larvae take shorter time to mature (Rueda *et al*., 1990), temperature in the range of 20 to 30°C and humidity greater than 60 percent are considered optimal for mosquito to survive long enough to acquire and transmit parasites (Devi and Jauhari, 2006). They can still breed in temperatures as low as 20^{o}C but cooler conditions will severely hamper the hatching of larvae. The minimum temperature for mosquito development is between 8-10^{o}C (Rueda *et al*., 1990).

Rainfall provides the breeding sites for mosquitoes and increases relative humidity necessary for mosquito survival, leading to increase in human biting rate. Humidity is one of the most important environmental factors affecting the mosquito distribution (Devi and Jauhari, 2008). The areas at lower elevation produced the greater number of species may be due to increased human disruption in those areas and mosquito diverse more between 500 to 900m (Devi and Jauhari, 2004). A decrease in the number of mosquito species at the higher elevation has already been reported (Scanlon, 1965; Basio *et al*., 1970; Bhat, 1975, Rajput and Singh 1988). The studies of Savage *et al.* (1990), Almiron and Brewer (1994), Rajnikant *et al.* (1996) and Reisen *et al.* (2006) demonstrated interspecific associations among mosquitoes and a correlation with physico-chemical and biological composition of mosquito breeding waters. Mosquitoes species differ in the type of aquatic habitats, they prefer for oviposition based on location, the physico-chemical condition of the water body, and the presence of potential predators (Shililu *et al*., 2003; Piyaratnea *et al.,* 2005). *Anopheles* mosquito has been found to use fresh water habitats for breeding (Tiimub *et al*., 2012). Water of a near neutral pH of 6.8 – 7.2 is preferable for breeding of many species of mosquitoes as weakening of the egg shells for

the first instar larvae stage to emerge (Okogun *et al*., 2003) whereas few species breed in tree holes or the leaf axils of some found to be most optimal for the weakening of the egg shells plants (CDC, 2004). Larvae of *Anopheles* mosquitoes in clear water of suitable temperature and nutrient conditions have been found to thrive in aquatic bodies such as fresh composition (Russel, 1999). However, edges of streams and rivers leads *Anopheles* species larval deaths due to reduction in small, temporary rain pools (Centers for Disease Control and Prevention, 2007) as well as high water current and flooding have been reported to lead to *Anopheles* species larval deaths due to reduction in oxygen tension causing physical harm to the larvae (Okogun, 2005). Physico-chemical factors that influence oviposition, survival, and the spatio-temporal distribution of important disease vector species include salts, dissolved organic and inorganic matter, turbidity, presence of suspended mud, presence or absence of plants, temperature, light and shade and hydrogen ion concentration (Mogi, 1978; Amerasinghe *et al.,* 1995; Gimnig *et al*., 2001). Several studies have examined the relationship between habitat characteristics and larval abundance. In Sri Lanka, *Anopheles culicifacies* was positively associated with light and vegetation and negatively associated with the presence of potential predators, while *Anopheles varuna* was positively associated with a variety of aquatic fauna (Piyaratnea *et al.,* 2005). In Venezuela, salinity and dissolved oxygen were associated with the spatial distribution of *Anopheles aquasalis* and *Anopheles oswaldoi. Culex quinquefasciatus* larvae, in Peninsular Malaysia, were most abundant in polluted drains containing 1.0 to 2.0 g/liter of dissolved oxygen, 1.0- 2.4 g/liter of soluble reactive phosphate, and 0.1-0.9 g/liter of ammonical nitrogen (Hassan *et al*., 1993). Studies of Kengluecha *et al*. (2005) in Thailand stated that, water hardness was probably responsible for the dominance of *Anopheles minimus* while negative relationship between pH, *Anopheles dirus* larvae was found in habitats with lower pH values especially in the

ground pools. *Anopheles vagus* prefer a higher pH for rice paddy field whereas *Anopheles campestris* prefer a higher concentration of dissolved oxygen in the swamp.

1.5. Insecticide resistance

Resistance to insecticides is the development of ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a population of the same species (WHO, 1975). Most insecticide resistance mechanisms can be divided into two groups, target-site resistance (sensitivity change or mutations in the target genes) and insecticide metabolism (alterations in the levels or activities of detoxification proteins). These mechanisms alone or in combination confer the resistance, sometimes at an extremely high level, to all of the available classes of insecticides. Besides the direct detoxification, an indirect way of insecticide resistance is a sequestering strategy. In this strategy, a metabolic enzyme acts as a ligand binding protein (Grant *et al.,* 1989) which rapidly binds to the insecticides resulting in a slowdown of the turnover rate (Hemingway, 2000) and decreasing the levels of free insecticides that prevents them from reaching and affecting the target sites. Many insecticides such as DDT and permethrin also influence insect's behavior by reducing the rate of mosquito entry into houses, increasing the rate of early exit from houses, and inducing a shift in biting times (Lines and Nassor, 1991; Mathenge *et al*., 2001). Some mosquitoes have also evolved thicker or altered cuticles for reducing the penetration of insecticide (Stone and Brown, 1969; Apperson and Georghiou, 1975).

1.6. Target-site resistance

The organochlorine, organophosphorus, carbamates, and pyrethroid insecticides all target the nervous system of insect species (acetylcholinesterase, GABA receptors, and voltage-gated sodium channels). Non-silent point mutations within these structural genes are the most common cause of target-site resistance. When selection of the mutations occurs, the resultant amino acid change must reduce the binding of the insecticide without causing a loss of primary function of the target site. Therefore the number of possible amino acid substitutions is very limited. Hence, identical resistance-associated mutations are commonly found across highly diverged taxa. The degree to which function is impaired by the resistance mutation is reflected in the fitness of resistant individuals in the absence of insecticide selection (Gulsiri, 2006). This fitness cost has important implications for the persistence of resistance in the field.

1.7. Insecticide metabolisms

Three major enzyme groups are responsible for metabolic based resistance to organochlorines, organophosphates, carbamates, and pyrethroids. The first one is esterases that are often involved in organophosphate, carbamate and to a lesser extent, pyrethroid resistance. The second is P^{450} monooxygenases, which are involved in the metabolism of pyrethroids, the activation and/or detoxification of organophosphorus insecticides and to a lesser extent, carbamate resistance. The last one is glutathione transferase (GST) which has been shown to have DDT-dehydrochlorinase activity in *Anopheles* and *Aedes* mosquitoes (Grant *et al*., 1989; Prapantharada *et al*., 1995) as well as role in organophosphate resistance in housefly, *Musca domestica* (Clark *et al.,* 1986).

1.8. Glutathione S-transferases

The glutathione S-transferases (GSTs) are members of a large family of multifunctional intracellular enzymes involved in the detoxification of endogenous and xenobiotic compounds via glutathione conjugation, dehydrochlorination, glutathione peroxidase (GPx) activity or passive/sacrificial binding (Hayes and Wolf, 1988; Mannervik and Danielson, 1988; Pickett and Lu, 1989; Yang *et al.*, 2001). GSTs can also serve as non-enzymatic binding proteins (known as ligandins) participating in the intracellular transport (Listowsky *et al.,* 1988) and signalling processes (Adler *et al*., 1999; Cho *et al*., 2001). This diversity of enzymatic and non-enzymatic functions is related to the genetic capacity to encode different GST isoforms by most organisms. Elevated levels of GST activity have been found to be associated to insecticide resistance in many insects. One or more GSTs have often been implicated in the resistance to organophosphates (OPs) in the house-fly, *Musca domestica* (Wei *et al.,* 2001), organochlorine 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl) ethane (DDT) in the fruit fly, *Drosophila melanogaster* (Tang and Tu, 1994) and more recently also reported in pyrethroid resistance strains of plant hopper, *Nilaparvata lugens* (Vontas *et al*., 2001). In mosquitoes, the metabolic resistance based on GST is the major mechanism of DDT-resistance (Hemingway and Ranson, 2000). GSTs are also involved in intracellular transport, biosynthesis of hormones and protection against oxidative stress. In addition, they contribute to the removal of toxic oxygen free radical species produced through the action of pesticides. They have peroxidases (Mannervik and Danielson, 1988; Zhao *et al*., 1999) and isomerase activity (Mannervik and Danielson, 1988), they can inhibit the Jun N-terminal kinase (thus protecting cells against H_2O_2-induced cell death) (Yin *et al*., 2000) and they are able to non-catalytically bind a wide range of endogenous and exogenous ligands (Bhargava *et al.,* 1978; Dulhunty *et al*., 2001). Some relevant aspects of the genetic

organization and metabolic function of mosquito GSTs in insecticide resistance have been reviewed (Hemingway, 2000; Hemingway and Ranson, 2000; Hemingway *et al*., 2004; Enayati *et al*., 2005), and also a compendium about particular aspects of GST including a brief overview of GSTs in mosquitoes has been published (Ranson and Hemingway, 2005).

1.9. GST structure

The vast majority of GSTs are cytosolic dimeric proteins comprising two subunits each around 24–28 kDa in size. The polypeptide chain of each monomer folds into two domains joined by a variable linker region. The N-terminal domain (residues 1–80) consists of four beta sheets and three flanking alpha helices, which are highly conserved and contain the majority of residues involved in the binding of glutathione (the G-site). The larger C-terminal domain has the variable hydrophobic substrate binding site or H-site and consists of a variable number of alpha helices and is more variable in structure (Armstrong, 1997). Although each monomeric active site functions independently, the quarternary structure is essential for activity (Mannervik and Danielson, 1988). Dimerization of GSTs allows the construction of a fully functional active site and also contributes to stabilization of subunit tertiary structure and quarternary structure (Dirr *et al*., 1991). Another reason for the dimerization of GSTs is that the association between two subunits may generate an intrasubunit-binding site for large bulky ligands that would not be able to bind to a monomeric GST (Hornby *et al*., 1994; Sayed *et al.,* 2000).

1.10. GST classification and organization

Most organisms posses multiple GSTs belonging to two or more classes. This variability has been achieved by extensive duplication and diversification of this supergene family. Curently more than 40 GST genes have been detected and grouped into at least 13 diferent classes (Alpha, Mu, Pi, Theta, Sigma, Zeta, Kappa, Omega, Phi, Tau, Beta, Delta, and Epsilon) based on their amino acid sequence identities, immunological properties, substrate specificities and tertiary structures (Ding *et al*., 2003). Generally, the intra-class GSTs have greater than 40% amino acid sequence identity whereas the inter-class GSTs have amino acid sequence identity less than 30% (Chelvanayagam *et al.,* 2001). Insect cytosolic GSTs were initially assigned numbers according to their order of elution from the various purification procedures employed or isoelectric points (Clark *et al*., 1986; Prapanthadara *et al*., 1993). Later two immunologically distinct classes of GSTs were recognized in houseflies and designated as class I and class II (Fournier *et al*., 1992). The class I insect GSTs are encoded by a single gene in all species studied to date (Beall *et al*., 1992,) although two distinct transcripts are produced by alternative splicing of the *Anopheles gambiae* class II gene (Ding *et al*., 2003). The class I insect GSTs, in contrast, are encoded by a multigene family in *Anopheles* mosquitoes, *D. melanogaster* and *M. domestica* (Toung *et al*., 1990; Zhou *et al*., 1998). As the volume of insect sequence data increased, additional GSTs were identified that clearly did not fit within class I or II. Phylogenetic comparison of insect and mammalian GST genes showed that the insect class II GSTs are orthologous to the Sigma GST class found in a diverse range of species from nematodes to mammals. In contrast, the class I GSTs is unique to insects and was re-named Delta GSTs. A second large class of GSTs, the Epsilon class, is also restricted to insects. The Delta and Epsilon GST classes have expanded independently in *D. melanogaster* and *Anopheles gambiae*, suggesting that these enzymes play

important roles in the adaptation of these species to their specific environments (Ranson *et al*., 2002). The majority of the remaining cytosolic insect GSTs are members of the Zeta, Theta and Omega classes (Board *et al*., 2000). The relatively high degree of conservation of these GST genes across taxa suggests that they play essential steps in conserved physiological pathways. In an African malaria mosquito, *Anopheles gambiae,* at least six classes of insect GSTs have been identified and found in several large clusters on all three chromosomes (Ding *et al.,* 2003). The Delta and Epsilon classes found exclusively in insects are the largest classes of insect GSTs. Members of both classes have been implicated in resistance to all the major classes of insecticide. The other anopheline GSTs belong to the Omega, Sigma, Theta and Zeta classes (Wang *et al*., 1991; Huang *et al.,* 1998; Ranson *et al*., 2001; Vontas *et al*., 2002; Ortelli *et al*., 2003).

1.11. GST detoxification functions

The GST-based detoxification of both endogenous and xenobiotic compounds can be in a direct way (phase I metabolism) or by the catalysis of reactive products formed by other enzymatic detoxification systems (phase II metabolism) (Yu, 1996; Sheehan *et al.,* 2001). In a reaction of conjugation, the active site residue interacts with the GSH sulphydryl group (-SH), to generate the catalytically active thiolate anion (GS-). This nucleophilic thiolate anion is then capable of attacking the electrophilic centre of any lipophilic compound to form the corresponding GS-conjugate (Jakoby and Ziegler; 1990, Armstrong, 1991). The conjugation neutralizes the electrophilic sites of the substrate, leading to its detoxification by the elimination of highly reactive electrophiles or rendering the product more water soluble and therefore more readily excretable from the cell (Habig *et al*., 1974; Hayes and Wolf, 1988). These conjugates

are eliminated from the cell *via* the glutathione S-conjugate export pump (phase III detoxification system) (Sheehan *et al*., 2001).

1.12. Insecticides Detoxification of GST

1.12.1. Organophosphates

Detoxification occurs by the conjugation of GSH to OP insecticides via two distinct pathways: an O-dealkylation or O-dearylation conjugation. In O-dealkylation the GSH is conjugated with the alkyl portion of the insecticide, while in the O-dearylation the GSH reacts with the leaving group. The reactions have been reported in housefly, *M. domestica* (Ugaki *et al*., 1985; Oppenoorth *et al*., 1979) and in diamondback moth, *Plutella xylostella* (Chiang and Sun, 1993) and verified by the use of recombinant GST enzymes in both species (Huang *et al*., 1998). The GSTs often act as a secondary resistance mechanism in conjunction with a Cytochrome P^{450} or esterase-based resistance mechanism (Hemingway *et al*., 1991). Most OP insecticides are usually applied in the non-insecticidal phosphorothionate form and are activated to the insecticidal organophosphate form (oxon analogue) by the action of cytochrome P^{450} within the insect. These oxons are more neurotoxic (potent acetylcholinesterase inhibitors) than their thionate analogues. Detoxification of the oxon analogues of fenitrothion has been reported in *Anopheles subpictus* (Hemingway *et al*., 1991). This co-operative enzyme system of detoxification would be more rapid and efficient than independent mechanisms and it is therefore important in insecticide resistance (Bogwitz, 2005).

1.12.2. Organochlorines

The GSTs catalyzed two detoxification reactions of halogenated hydrocarbons: dehydrochlorination and GSH conjugation (Tang and Tu, 1994). The DDT dehydrochlorination is the major route of detoxification for this insecticide (Hayes and Wolf, 1988) and probably the most common DDT resistance mechanism in mosquitoes (Brown, 1986, Hemingway, 2000). In the glutathione-dependent DDT dehydrochlorination, the GS- generated in the active site acts as a general base and removes hydrogen from DDT resulting in the elimination of chlorine to generate the non-toxic DDE (1,1-dichloro-2,2-bis-[*p*chlorophenyl] ethane). In this reaction the GSH levels do not change at the end of the reaction (Lipke and Chalkley, 1962) due to the GSH regenerated acting as a cofactor rather than a conjugate (Clark and Shamaan, 1984). An increased rate of glutathione-dependent dehydrochlorination confers resistance to DDT in *Aedes aegypti* (Grant *et al*., 1991; Lumjuan *et al*., 2005), *Anopheles dirus* (Prapanthadara *et al.,* 1996) and *Anopheles gambiae* (Prapanthadara *et al*., 1993; Ranson *et al*., 2001; Ortelli *et al*., 2003). Another organochlorine insecticide like lindane is suggested to be detoxified initially by a dehydrochlorination reaction and subsequently by conjugation to glutathione (Tanaka *et al*., 1981) both reactions being catalyzed by GST (Clark *et al*., 1986; Bloomquist, 1998; Wei *et al*., 2001). However, the major routes of metabolism of lindane include dehydrochlorination by GST giving various chlorobenzenes, along with subsequent cytochrome P^{450}-mediated hydroxylation to yield several chlorophenols (Bloomquist, 1998).

1.12.3. Pyrethroids

GST's role in the detoxification of pyrethroids has been basically attributed to its capacity to reduce the peroxidative damage induced by pyrethroids, mainly by detoxifying lipid peroxidation products (Vontas *et al*., 2001). This evidence was suggested for a delta class GST

from a pyrethroid resistant strain of rice brown plant hopper, *Nilaparvata lugens* whose recombinants showed high peroxidase activity (Vontas *et al*., 2002) and recently reported for an epsilon class GST in *Aedes aegypti* mosquitoes (Lumjuan *et al*., 2005). Several GSTs which accepted an LPO product as substrate have also been reported in *D. melanogaster* (Singh *et al*., 2001; Sawicki *et al*., 2003). It is suggested that GSTs may also protect against pyrethroids toxicity in insects through a passive sequestration process (Kostaropoulos *et al*., 2001). The evidence of some GSTs binding to various pyrethroids has been reported in *Anopheles dirus* (Prapanthadara *et al*., 1996; Jirajaroenrat *et al*., 2001; Udomsinprasert and Ketterman, 2002). The use of GST inhibitors (eg. diethyl maleate) in pyrethroids resistant *Culex* strains suggests that GST-mediated metabolism has a relative contribution in pyrethroids resistance (Xu *et al*., 2005). While in the field, *Anopheles albimanus* populations and slight increases of GST activities under continuous PYR selection were also detected (Penilla *et al*., 2006).

1.13. Regulation of GST expresion

In non-insect species, many GST enzymes are differentially regulated in response to various inducers or environmental signals or in a tissue- or developmental-specific manner. A similar complex pattern of regulation is expected for insect GSTs. Two review articles have described the effect of various dietary compounds, insecticides and laboratory inducers on general GST expression (Clark *et al*., 1986; Yu, 1996). Now that the full extent of the GST family is known for two insect species, more specific studies can be conducted to determine the factors regulating expression of individual GST genes. Levels of GST activity vary throughout the life stages of insects. For example, in *Aedes aegypti*, total GST activity measured with 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloronitrobenzene (DCNB) increases during

larval development, reaching its peak in the pupal stage and declining in adults as they age (Hazelton and Lang, 1983). In a preliminary investigation of the expression profiles of *Anopheles gambiae* GSTs, transcripts were detectable for all but one of the genes in 1-day-old adults (Ding *et al*., 2003). No attempt was made to quantify the expression level in different developmental stages in *Anopheles gambiae* but it is apparent from studies in other insects that the levels of individual enzymes can fluctuate widely during the lifespan of an insect. For example, a Sigma GST from the spruce budworm, *Choristoneura fumiferana*, is expressed at very low levels in feeding larvae but high levels in diapausing larvae (Prapanthadara *et al*., 1996). Variations in the level of GST activity in different insect tissues have been reported in several species. In cases where the variation in activity is attributed to individual enzymes, such studies can provide valuable insights into the functions of different GSTs. Thus, the finding that Sigma GSTs from housefly and *Drosophila* were predominately located in the indirect flight muscles, in association with troponin H, suggested that the role of this GST class was structural rather than catalytic (subsequently, however, these GSTs have been found to play a very important role in protection against oxidative stress) (Singh *et al*., 2001). Although the significance of alternative splicing in regulating GST expression has not been fully investigated, four alternative transcripts of a Delta class gene and two of a Sigma GST have been detected in *Anopheles gambiae* (Ranson *et al*., 1998) and it is possible that different inducers or stress treatments may affect the alternative splicing. Most studies of GSTs suggest that regulation occurs at the transcriptional level. Several regulatory elements have been identified in the promoter regions of GSTs that may mediate their induction but the significance of these findings is unclear in the absence of functional studies. In *Aedes aegypti*, a mutation in a trans-acting repressor element is the proposed mechanism for the enhanced expression of a Delta class GST

in a DDT-resistant strain (Grant and Hammock, 1992). Genetic mapping of the major genes controlling GST-based DDT resistance in *Anopheles gambiae* also provided tentative evidence for a trans-acting regulator (Ranson *et al*., 2002) although in this species, mutations in promoter elements of the Epsilon GST cluster are also associated with resistance (Ranson *et al*., 2001). So, the potential role of GSTs in pesticide detoxification makes it an attractive target for structural analysis that will help in the understanding of the mechanisms of resistance to important pesticides and guide the design of novel inhibitors to overcome insecticide resistance. Furthermore, functional genomics approaches will contribute to understanding of the role of individual GSTs in insects and perhaps then the reason for the extensive diversity of this enzyme family will become clear.

1.14. P^{450} monooxygenases

Cytochrome P^{450}-dependent monooxygenases are an important and diverse family of numerous hydrophobic, heme-containing enzymes involved in the metabolism of endogenous and exogenous compounds. These enzymes are generally the rate-limiting step in the chain that leads to oxygen binding and electrons received from NADPH to introduce an oxygen molecule into the substrate (Singh *et al*., 2001). Diversity is conferred by the existence of multiple P^{450} isoforms, different expression patterns and wide substrate specificity (Feyereisen, 2005). Elevated monooxygenase activity is associated with pyrethroid resistance in *Anopheles stephensi, Anopheles subpictus, Anopheles gambiae* (Hemingway *et al*., 1991) and *Culex quinquefasciatus* (Singh *et al*., 2001). There are many reports demonstrating elevated P^{450} monooxygenase activities in insecticide-resistant mosquitoes, frequently in conjunction with altered activities of other enzymes such as the elevation of oxidized and esterase levels in

permethrin-resistant *Anopheles gambiae* from Kenya (Vulule *et al*., 1999) and the reports of oxidazed-based and esterase-based resistance mechanisms alone and in combination in permethrin-resistant *Anopheles albimanus* from Guatemala (Brogdon *et al*., 1999).

1.15. Esterase-Based Resistance

The esterase-based resistance mechanisms have been studied extensively at the biochemical and molecular level in mosquitoes. Work is in progress on related and distinct esterase resistance mechanisms in a range of *Anopheles* and *Aedes* species. Broad-spectrum organophosphate resistance is conferred by the elevated esterases of *Culex*. All these esterases act by rapidly binding and slowly turning over the insecticide: They sequester rather than rapidly metabolize the pesticide (Kadous *et al*., 1983). Two common esterase loci, *est-α* and *est-β*, are involved alone or in combination in this type of resistance in *Culex* (Vaughan *et al*., 1997). The classification of these esterases is based on their preferences for *α*- or *β*-naphthyl acetate, their mobility on native polyacrylamide gels and their nucleotide sequence (Hemingway *et al*., 2004). Smaller numbers of *Culex quinquefasciatus* populations have elevated est-*β* alone, elevated est-α alone or co-elevated *est-α* and *est-β* (De Silva and Hemingway, 2002). The superiority of insecticide binding in enzymes from the resistant strains suggests that there has been positive insecticide selection pressure to maintain elevation of favorable alleles of the esterases in insecticide-resistant insects. Metabolic studies on *Culex* homogenates suggests that an increased rate of esterase-mediated metabolism plays little or no role in resistance. One exception to this is *Culex tarsalis*, where two resistance mechanisms co-exist: one involving elevated sequestering esterases, the other involving non-elevated metabolically active esterases (Ziegler *et al*., 1987). In contrast to the situation in *Culex*, a number of *Anopheles* species have a non-elevated esterase

mechanism that confers resistance specifically to malathion through increased rates of metabolism (Malcolm and Wood, 1982; Hemingway *et al*., 1985; Boddington, 1992). Malathion carboxylesterase resistance has been found in *Anopheles culicifacies, Anopheles stephensi and Anopheles arabiensis* (Herath *et al*., 1987; Hemingway 1992). There are many reports of enhanced esterase activities in other mosquitoes, for example, in permethrin-resistant *Anopheles gambiae* (Vulule *et al*., 1999) and *Anopheles albimanus* (Brogdon *et al.,* 1990) and in resistant *Aedes aegypti* (Mourya *et al.,* 1993). The amplified carboxylesterases of some insects (the cattle tick *Boophilus microplus* and the peach-potato aphid *Myzus persicae*) do have activity against both pyrethroids and organophosphates (Devonshire *et al.,* 1982; Hernandez *et al*., 2002).

1.16. *In-silico* characterization of GST

Computational packages and online servers are the current tools used in the protein sequence analysis and characterization (Sivakumar *et al.,* 2007). The physico-chemical and the structural properties of the proteins are well understood with the use of computational tools. Today, number of computational tools has been developed for making predictions regarding the identification and structure prediction of proteins. The statistics about a protein sequence such as number of amino acid, sequence length, and the physico-chemical properties of a proteins such as molecular weight, atomic composition, extinction coefficient, GRAVY, aliphatic index, instability index, etc. can be computed by computational tools for the prediction and characterization of protein structure. The amino acid sequence provides most of the information required for determining and characterizing the molecule's function, physical and chemical properties. Sequence analysis and physicochemical characterization of proteins using

biocomputational tools have been done by many researchers and reported (Yuri *et al*., 2003; Courtney and Robert, 2004; Sivakumar, 2006).

Bioinformatics studies on three-dimensional structure of the target is essential for defining the active site and also for designing, improving, and docking of small ligands to the complex target protein. All cytosolic GSTs have the same basic protein folding, which comprises two domains. The N-terminal domain (domain I) adopts a *α/β* topology and provides the GSH-binding site (G-site) (Armstrong, 1997). It is currently believed that the residues which contribute to binding glutathione involve a network of specific polar interactions between GSH and G-site residues that are either conserved or conservatively replaced between classes. The C-terminal domain (domain II) is an all-helical structure and provides the structural element for recognition of the broad range of hydrophobic co-substrate [H-site (hydrophobic-substrate-binding site)], which lies adjacent to the G-site (Armstrong, 1997). It shows the greatest variability across the GST classes (Board *et al*., 2000) and helps to define the substrate selectivity of the enzyme. The active site residue tends to be highly conserved within GST classes, but differs between classes. In most mammalian GSTs, the active site residue responsible for the GSH thiol residue activation in catalysis appears to be a tyrosine (Sheehan *et al*., 2001), but in the delta and epsilon insect GST classes, this role is performed by a serine residue (Ranson and Hemingway, 2005; Udomsinprasert *et al*., 2005).

The undertaking of MSVBDCP (Mizoram State Vector Borne Diseases Control Programme) mainly focused on prevention of death and mobidity due to malaria by human treatment, vector control by means of IRS (Indoor Residual Spray) and personal protection by distributing LLINs; thereby neglecting effective and efficient surveillance system: entomological component *viz.*mosquito habitats and seasonal abundance and their role in diseases transmission as well as the tolerance of insecticides against diseases vectors. Understanding the relationship between habitats, environmental factors, distribution and relative abundance of vectors in the targeted areas is essential to know the status of the complexity of the resistance segregating in field populations for an efficient application of mosquito control methods as well as malarial cases reduction (Perera *et al*., 2008). Therefore, the present study was aimed to a) study the prevalence and abundance of mosquito species and their breeding habitats; b) establish the baseline susceptibility status against a commonly used synthetic insecticides; c) quantitative estimation of resistance enzymes (GST and esterases); d) expression of GST gene (resistant gene) and e) characterization of GST gene using bio-informatics tools on mosquito.

II. OBJECTIVE OF THE STUDY

III. OBJECTIVES OF THE STUDY

- To find out mosquito diversity and density in relation to their habitats.

- Susceptibility test of *Anopheles* species using insecticides and quantification of Esterase and GSTs enzyme activity.

- Characterization and expression profiles of Glutathione S-transferases (GSTs) gene from *Anopheles* species.

- Comparison of GSTs genes and isoforms in insects in relation to characterization and structure prediction of proteins using Bioinformatics tools.

IV. MATERIALS AND METHODS

III. MATERIALS AND METHODS

3.1. Study area

The study covered a major part of the six districts in Mizoram (between 2009 and 2011) including Aizawl (23°44' N, 92°42' E), Serchhip (23°16' N, 92°44' E), Mamit (23°55' N, 92°29' E), Lunglei (22°52' N, 92°43' E), Lawngtlai (22°18' N, 92°41' E) and Kolasib (23°13' N, 92°40' E) with the altitudinal variation of 54 - 1150 m. Priority on the sites of collection was mainly based on malarial prevalences and occurrences data obtained from Health Dept. Govt. of Mizoram. These were considered as probable mosquito larval habitats: (i) cemented pools (cemented walls), (ii) ponds, (iii) household water storage tanks (barrels), (iv) stagnant stream side pools, (v) temporary ditches, (vi) shallow pits and (vii) seapage pool, (viii) cattle sheds, (ix) human residents.

Figure 1. Map of Mizoram showing different districts.

2A. Mission veng river

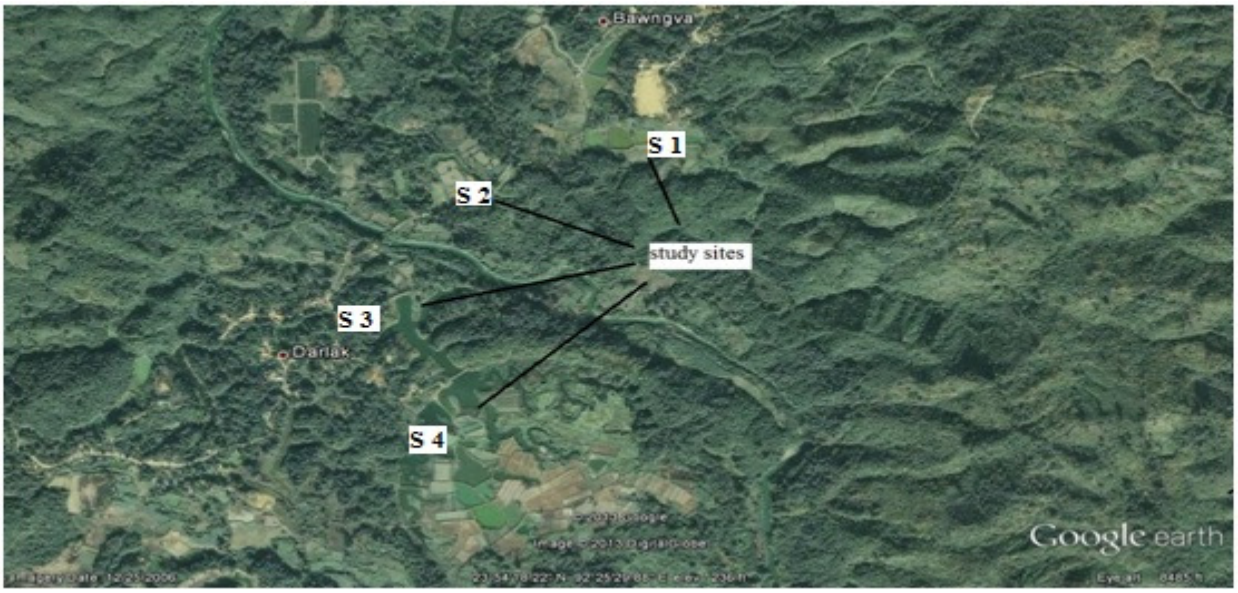
2B. Dinthar river

2C. Lengpui, fish ponds

2D. Ramrikawn pond

2E. Sihhmui semi-permanent pool

Figure 2A-E. Collection sites of mosquito vector complex from Aizawl district.



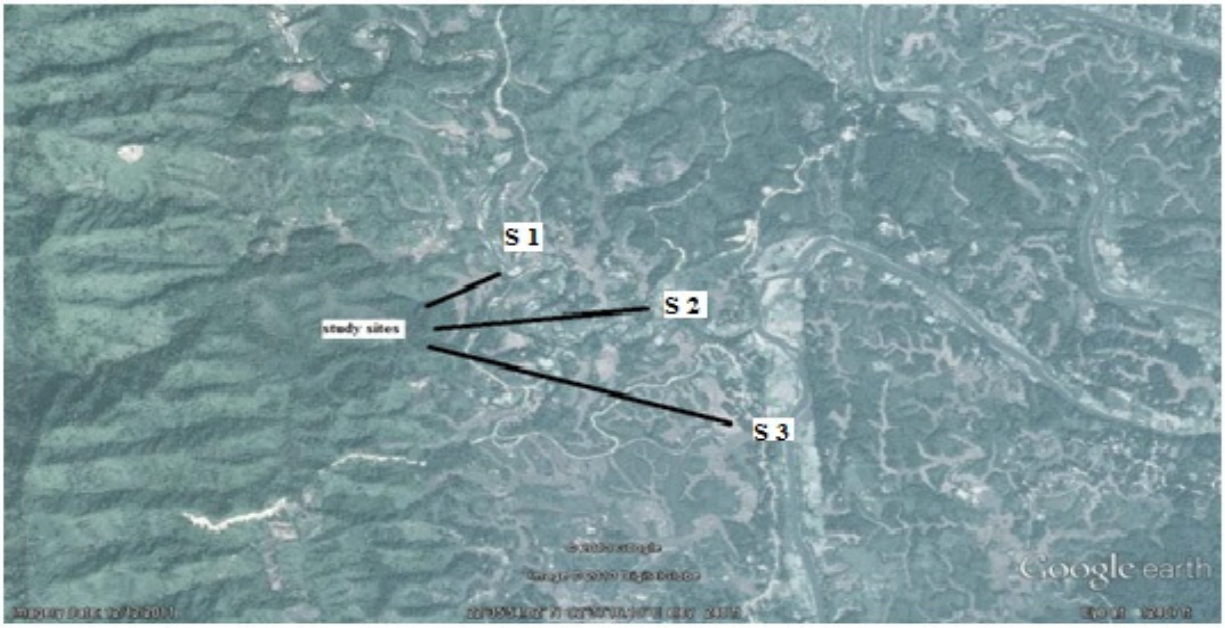
3A. Collection sites (S1-S4) at Mamit district

3B. Collection sites (S1-S3) at Kolasib district

3C. Collection sites (S1-S2) at Lawngtlai district

Figure 3A-C. Collection sites of mosquito vector complex from Mamit, Kolasib and Lawngtlai districts.

4A. Collection sites (S1-S4) at Thenzawl (Serchhip) district



4B. Collection sites (S1-S3) at Chawngte (Lunglei) district

Figure 4A-B. Collection sites of mosquito vector complex from Thenzawl (Serchhip district) and Chawngte (Lunglei district).

3.2. Diversity, distribution and population survey of Mosquito

3.2.1. Method of sampling and collection of Mosquito

3.2.1.1. Larva collection (scoop-net method)

The water bodies (ponds, ditches, pools, river beds, tree holes, rock holes, tanks and containers) were surveyed and subsequently sampled, collection of immature mosquitoes was also made on the same day (8:00 am – 3:00 pm) by the scoop-net method (WHO, 1975), with a larval net of a fine mesh net mounted to a iron handle (25 cm diameter), plastic tub of different sizes, plastic dipper and dropper (21 - 38^{o}C; 25 - 98% RH). Larvae collected in the field were sorted and segregated depending on Anopheline and Culicine larvae. It was then immediately carried to laboratory for further analysis.

3.2.1.2. Adult collection (hand collection and light trap) and preservation

Adults were collected at dusk and midnight (4:00 – 8:00 pm; 12:00 – 2:00 am) using electrical mosquito bat (commercially available), hand collection (WHO, 1975) which consisted of a 250 ml glass jar and cotton moisten with chloroform kept at the base of the jar and CDC (Center for Disease Control) light trap from both indoor and outdoor. Death and paralyzed collected adults were immediately transferred to 1.5 ml micro-centrifuge tube that contained silica gel and cotton. In laboratory, collected specimen were indentified, labelled and stored at 4^{o} C till further analysis.

3.2.1.3. Identification of mosquito

Morphological identification of mosquito was done on adult female taking colour pattern of wing, palpi and leg as identification characters using dissecting light microscope and hand lens. The identification keys followed the illustration of Das *et al*. (1990), Glick (1992), Reuben *et al.* (1994), Nagpal and Sharma (1995), Oo *et al*. (2005).

3.2.2. Seasonal Variation and relative abundance

To assess the temporal variation of the mosquitoes, Two-way ANOVA was conducted to justify difference in abundance of mosquitoes over time. One-way ANOVA followed by Tuckey's test was performed to find out significant difference in population of different species of mosquitoes. Also, Shannon-Wiener diversity index ($H' = -\sum_{i=1}^{R} p_i \ln p_i$) was calculated to noted the variation in mosquito temporally. All the calculations were performed using commercially available GraphPad instat Version 3.0 (GraphPad software inc., San diego, CA) and PAST 1.86b.

3.2.2.1. Distribution and Density of sampled Mosquito

Distribution and density dynamics of mosquito populations in the sampling sites were analyzed using the following factors (Rydzanicz and Lonc, 2003):

Distribution was determined as the percent of sampling sites in which a species was noted, according to the formula:

$$C = \frac{n.}{N} 100\%$$

where:

C - Distribution

n - Number of sites of the species
N - Number of all sites.

The following distribution classes were adopted (Dzieczkowski, 1972):

C1. Sporadic appearance (constancy 0-20 %)
C2. Infrequent (20.1-40%)
C3. Moderate (40.1-60%)
C4. Frequent (60.1-80%)
C5. Constant (80.1-100%).

Density was expressed as percent of specimens of the species in the whole sample according to the formula:

$$D = \frac{l}{L} \cdot 100\%$$

where:

D - Density, l - Number of specimens of each mosquito species, L- Number of all specimens.

The following density classes were accepted:

Satellite species (D < 1%)
Subdominant species (1< D <5%)
Dominant species (D > 5%).

3.3. Mosquito Larva and their breeding habitats association

Assessment has been done in relation to mosquito species and their breeding habitats preferences (Devi and Jauhari, 2007). The collected data with respect immature mosquitoes were analyzed using the following steps - i) listing of operative taxonomic units, ii) development of basic matrix of data and iii) calculation of similarity for each pair of mosquito species.

Since the main purpose was to group mosquito species with similar breeding features, the operative taxonomic units chosen were the mosquito species collected. The recorded breeding habitats and physical characters of breeding grounds were analyzed to identify common patterns

of immature stage habitats where different mosquito species were collected. Both quantitative (water depth) and qualitative (natural / artificial, permanent / temporary, shady / lighted, water movement, vegetational condition and turbidity) characters were codified as 1/0 (= presence/absence). A basic matrix of data was developed in tabular form on the basis of codified data, consisting of rows for mosquito species and columns for positive breeding habitats characters and its water quality. Values within the cells represent 1 or 0 if each character was recorded for each species or not, respectively. These data were used to analyze and calculate the similarity for all possible pairs of operative taxonomic units. The similarity for all possible combinations of mosquito species pairs (operative taxonomic units) was calculated using the coefficient of association and thereafter the similarity matrix was developed. Operative taxonomic units were grouped on the basis of similarity using cluster analysis (NTSYS-pc version 2.20f software package).

3.3.1. Interaction of mosquito species and breeding habitats with water quality

Water samples were taken from the different positive breeding habitats (every two months interval) such as ponds, river beds, ditches and pools within Aizawl district during 2009-2011. It was then immediately carried to laboratory for analysis of different parameters and to find out the best predictor for larval abundances. The water quality analysis was done as per Trivedy *et al.* (1987) and American Public Health Association (1992). Preparation of chemicals/reagents for water quality analysis were shown in Annexture 1.

3.3.1.1. Temperature and pH

Temperature of the water body in the breeding habitats was measured by Thermometer (Mercury in glass thermometer) in the collection sites. pH was measured by pH paper in the field and Eutech instrument pc 510 instruments in laboratory.

3.3.1.2. Phosphate estimation (American Public Health Association, 1992)

Preparation of standard graph for Phosphate:-

Different concentration of standard phosphate solutions *viz.* 0. 5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml were taken in separate beakers. Water was added to make the volume 50 ml. 2 ml of ammonium molybdate and 5 drops of $SnCl_2$ was added to it. OD was taken at 690nm. A graph was plotted against different concentrations of phosphate from the OD value.

Estimation of Phosphate concentration was done (Stannous chloride reduction method) by taking 50 ml of water sample was taken in beaker and filtered it. 2 ml of ammonium molybdate and 5 drops of $SnCl_2$ was added- a blue colour developed. Simulteneously, a blank was prepared by adding the same amount of reagents and 50 ml of water. It was incubated in room temperature and then the OD was taken at 690 nm.

Calculation:

From the standard graph-

'x' value of OD = ***'y'*** value of PO_4 gives the value of *'z'*

OD value of unknown sample = ***'v'***

Final value = ***'v'*** x *'z'* mg of PO_4

3.3.1.3. Dissolved oxygen estimation (American Public Health Association, 1992)

Water sample was fixed in the field for estimating the amount of dissolved oxygen present in the water by 300-ml glass Biological Oxygen Demand (BOD) stopper bottle. In laboratory, 2ml of manganese sulphate and 2 ml of alkali-iodide-

azide reagent was added to the collection bottle. The sample was mixed by inverting several times and checked for air bubbles. 2 ml of concentrated sulfuric acid was added via a pipette held just above the surface of the sample. Carefully stopper and solution was added as indicator. It was then titrated against sodium thiosulphate to a pale straw color as end point.

Calculation:

$$DO\ (mg/L) = 8\ \frac{x\ 1000\ x\ N\ x\ Vol.\ of\ titrant}{Sample\ volume\ (ml)}$$

Where,

N is the normality of the solution (0.025N)

3.3.1.4. Alkalinity estimation (American Public Health Association, 1992)

25 to 50 ml of water sample was taken in a conical flask. 2 to 3 drops of phenolphthalein Indicator was added to it. If it turns pink (pH > 8.3), it was then titrated against 0.02 N H_2SO_4 (2.8 ml conc. sulphuric acid was diluted to 1L to make 0.1 N, 0.1N solution was approximately diluted to 1L to make 0.02 N H_2SO_4) to disappearance of the colour. Volume of titrant (ml) used was recorded.

Calculation:

$$Phenolphthalein\ alkalinity,\ mg\ CaCO_3\ /L = \frac{A\ x\ N\ x\ 1000}{ml\ of\ sample}$$

where,

A = ml of titrant used to phenolphthalein end point
N = Normality of titrant (0.02N).

3.3.1.5. Hardness estimation (American Public Health Association, 1992)

25 ml of water sample was taken in conical flask. 1 ml of Ammonia buffer solution (pH 10) was added to it and then shaken thoroughly. Two drops of

Eriochrome black T indicator was added and the solution turns into wine red in colour. It was then titrated against Standard EDTA titrant (0.01 M) till the solution turned from wine red to blue colour as end point.

Calculation:

$$\text{Total Hardness (EDTA) in mg/L as CaCO /L} = \frac{\text{ml of titrant used x 1000}}{\text{ml of sample}}$$

3.3.1.6. Chloride estimation (American Public Health Association, 1992)

To 50 ml of water sample in conical flask, 1 ml K_2CrO_4 indicator solution was added (50 g K_2CrO_4 was dissolved in a distilled water, $AgNO_3$ solution was added until a definite red precipitate was formed. It was allowed to stand for 12 h, filtered and diluted to 1 L of distilled water) and titrated against 0.0141N $AgNO_3$ titrant (2.395 g of AgNO3 dissolved in distilled water and dilute to 1L of H_2O) to a pinkish yellow colour as end point.

Calculation:

$$\text{Cl in mg /L} = \frac{\text{A x N x 35.46 x 1000}}{\text{ml of sample}}$$

where,

A = ml of titrant

N = Normality of $AgNO_3$ (0.0141N)

3.3.1.7. Total dissolved solids estimation (American Public Health Association, 1992)

Clean conical flask was taken and weighted it in electronic balance. 250 ml of water sample was poured into conical flask, heated in hot plate and allowed to dry. Dried conical flask was then cooled in room temperature for about ½ hrs. It was then weighted again in electronic balance and the reading was noted.

Calculation:

$$\text{TDS in mg/L} = \frac{\text{A - B x 1000}}{\text{Vol. of sample}}$$

Where,

A = Final weight of beaker

B = Initial weight of beaker

3.4. Insecticidal Bioassay

Susceptibility tests was carried out in three replicates using DDT (50% effective concentration) and Deltamethrine (2.5% active ingredient w/w) obtained from Department of Health Services, Govt. of Mizoram.

3.4.1. Maintenance of mosquito and preparation of test concentrations (WHO, 1981)

Bioassay was conducted on field collected population. Larvae collected from the field were immediately carried to laboratory (25±3^{o} C temp, 50-85% RH). Small amount of ground fish food was given as the supplement and 3rd instars larvae were used for bioassays. Adult susceptibility tests were done on early adults that emerged from larvae in laboratory. Insecticides of different concentrations was prepared from stock concentration: DDT *viz.*0.004, 0.02, 0.10, 0.50 and 2.5 ppm for larval bioassay and 0.25, 0.50, 1.0, 2.0 and 4.0% for adults bioassay, Deltamethrin *viz.* 0.002, 0.004, 0.008, 0.01 and 0.03 ppm for larval bioassay and 0.004, 0.006, 0.008, 0.01 and 0.025% was used for adults bioassay.

3.4.2. Larval susceptibility assay

Susceptibility tests were carried out in three replicates as per WHO (1981) protocol. *Anopheles vagus* and *Anopheles barbirostris* were used for the assay. Insecticides of different concentrations *viz*. DDT (0.002-2.5 ppm) concentration and deltamethrin (0.002-0.03ppm) concentration (100 ml) were exposed to three replicates of thirty 3rd instars larvae in disposable cups. The morbid larvae were continually revived with a needle. Control experiments were also done by 100 ml of dechlorinated water. After 24 hours, mortality was

counted. The concentration that yielded up to 90 % mortality was used to determined the lethal concentration of 50 % (LC_{50}).

3.4.3. Adults susceptibility assay

Adult Insecticide bioassays were conducted by means of tarsal contact exposure to insecticide-impregnated papers as per WHO protocol (1963). Rectangles of Whatman-No.1 filter papers (12 cm × 15 cm) was used for insecticide impregnation. DDT of different concentrations (0.25% - 4%) and deltamethrin (0.004 - 0.25 %) of 0.7 ml each were mixed with an equal volume of acetone (0.7 ml) and the mixture was spread uniformly on the filter paper (Perera *et al*., 2008). Batches of 10–20 early adults female mosquitoes (depending on the availability) were exposed to insecticide impregnated papers for one hour, dead mosquitoes were counted after a recovery period of 24 hours. Papers impregnated with the carrier (oil) and acetone was used as controls. The concentration that yielded up to 98% mortality was used to determined the lethal concentration of 50 % (LC_{50}).

The LC_{50} values for the two insecticides were calculated by Probit analysis. The percent mortality was corrected using Abbott's formula when the control mortality was less than 20 percent.

$$\text{Abbott's formula} : \frac{\%\ \text{test mortality} - \%\ \text{control mortality}}{100 - \%\ \text{control mortality}} \times 100$$

3.5. Biochemical analysis of insecticides treated samples (WHO, 1998)

3.5.1. Preparation of mosquito for quantitative enzyme assays

Enzyme assays was done as per WHO protocol (1998). Different concentration of insecticides treated mosquitoes (larvae and adults) which were alive after treated (stored at -20^{o}C) was homogenized in -20^{o}C cryo-box. 200 µl distilled water was added to it. It was spun at 14,000 rpm for 30 seconds; the supernatant was used as enzyme samples then stored at -20^{o}C.

3.5.2. Protein assay

Total protein estimation followed the procedure of Lowry's *et al.* (1951). A standard protein of Bovine Serum Albumin (BSA) of 200µl/ml stock concentration was pipette out as 100, 200, 400, 600, 800 and 1000 µl. The volume was made to 1 ml by adding sterile water to each test-tube. For blank, 1 ml of sterile water was used. 5 ml of the reagent B (Appendix II) was added to all the test-tubes. The solution was mixed well, shaken thoroughly and incubated at room temperature for 10 minutes. Again, 500 µl of reagent C (Appendix II) was added and mixed well. A blue colour solution was developed and it was again incubated at room temperature for another 30 minutes. Optical Density (OD) was measured at 660nm in spectrophotometer.

3.5.3. Standard (α-and β-) Napthol assay

α-napthol and β-napthol of 200µl/ml stock concentration was pipette in 100, 200, 400, 500 and 800 µl into test-tubes. The volume was made to 1 ml by addition of 0.02M PBS (pH 7.2) to each test-tubes and the blank contained 1 ml of 0.02M PBS (pH 7.2). 50 ml of Fast blue stain was added to each test-tubes and incubated at room temperature for 5

minutes. OD was read at 570 nm. Two standard curved were made for α-napthol and β-napthol (WHO, 1998).

3.5.4. Naphthyl Acetate assay for Esterase

200µl of α-/β- Naphthyl Acetate was added to 20µl of homogenate and incubated st room temperature for 15 minutes. The blank contained 20µl of distilled water. 50 µl of Fast blue stain was added and further incubated for another 5 minutes. 2860 µl of 0.02M Phosphate buffer (pH 7.2) was added to increased the volume required by spectrophotometer used. OD was then read at 570 nm (WHO, 1998).

Calculation:

The total protein value was first read from BSA standard curve and multiplied by 2 times, which gave the amount of protein expected in 20 µl homogenate and was recorded in µg to give the value of ***'x'***. The OD value obtained from both α- and β- naphthyl acetate assays were multiplied by 13 (it was 13 times dilution) and was then read from the corresponding α-/β- naphthol standard curves. This value was divided by 15 (no. of minutes incubated before the satin was added) to give the value of ***'y'***. Thus, ***'y'*** value was divided by the value of ***'x'*** and timed this by 1000 to give the final value as α-/β-naphthol/min/mg protein (WHO, 1998).

3.5.5. Assay for Glutathione-S-Transferase

10 µl of homogenate was mixed with 200 µl of chlorodinitrobenzene-Reduced Glutathione (CDNB-GSH) and incubated for 20 minutes. The blank contained 10 µl of distilled water. 2940 µl of of 0.02M Phosphate buffer (pH 7.2) was added to increase the

volume required by spectrophotometer used and mixed thoroughly. It was incubated for 20 minutes in room temperature. OD was then read at 340 nm (WHO, 1998).

Calculation:

The activity of GST was calculated assuming that absorbance followed Beer-Lambert's law: ***A=εcl,*** where ***'A'*** is the absorbance, ***'ε'***is extinction coefficient of the product of reaction of CNDB valued $4.39mM^{-1}$, 'c' is the concentration and ***'l'*** is the path length (of the cuvette used in spectrophotometer) which was 1 cm to give an ***'x'*** value. The value of ***'x'*** was again multiplied by 14 (times 14 dilution) to give value of ***'y'.*** The value of ***'y'*** was divided by 20 (no. of minutes for incubation) and divided by BSA total protein value to give value of ***'z'.*** The value of ***'z'*** was then multiplied into 1000 times to give final value as µMoles/min/mg protein (WHO, 1998).

3.6. Preparation and extraction of total RNA from *Anopheles* species

The procedure followed for preparation and extraction of total RNA was as described by Sambrook *et al*. (1989) and Simms *et al*. (1993). 0.5 µl of Diethylpyrocarbonate (DEPC) was added to 100 ml sterile water (volume was also made to 1000ml) and stirred overnight with a magnetic stirrer to make 0.1% DEPC water. Pipette tips, Pipette boxes, tubes (1.5 ml and 0.2 ml) and homogenizers were soaked overnight in 0.1% DEPC water and autoclaved twice for 15 minutes and then dried in oven. Mosquito was homogenized in -20^{o}C mini cooler. 460 µl Trisoln ^{TM} (GeNei) was added to the sample, mixed by gentle tapping and incubated at 28^{o}C for 10 minutes and spun at 12,000 rpm for 10 minutes at 4^{o}C. The supernatant was transferred into a fresh sterile 1.5 ml micro-centrifuge tube. 92 µl chloroform was added to the supernatant, incubated at 28^{o}C for 5 minutes and spun at

12,000 rpm for 15 minutes at 4°C. 60% (approx. 270 µl) of the aqueous solution was pipette out and pour in 230 µl isopropanol and incubated further for 10 minutes at room temperature and again spun at 12,000 rpm for 15 minutes. The pellet was washed with 460 µl of 70% alcohol and centrifuged at 5,000 rpm for 5 minutes and air-dried. RNA was re-dissolved in 20 µl nuclease free water and incubated at 55°C for 10 minutes. The RNA samples were quantified using Biophotometer plus™ (Eppendorf, Germany) and the samples were diluted to final concentration of 100 ± 4ng/µl. 5µl aliquots were made and stored at -20°C.

3.6.1. cDNA synthesis from total RNA

The cDNA was synthesized using RevertAid™ First strand cDNA synthesis kit (Fermentas) following the manufacturer's protocol. 1 µl of DNase™ (GeNei) was added to the extracted RNA samples and incubated at 37°C for 15 minutes for the degradation of the DNA contaminants and then at 75°C 15 minutes for degradation of DNase. The sample was quantified using Biophotometer plus ™ (Eppendorf, Germany). 10 ± 5 ng/µl of RNA was used for cDNA synthesis. 1 µl of RNA sample and 1 µl of oligo (T)™ primer was mixed and the volume was made to 12 µl with DEPC treated water. The mixture was incubated at 65°C for 5 minutes on ice. 4 µl 5X Reaction buffer, 1 µl Ribolock ™ RNase inhibitor, 2 µl of 10mM dNTP mix and 1 µl Reverse transcriptase was added to the mixture, mixed by gentle tapping and centrifuge using Mini-spin™ (GeNei). The solution was incubated at 43°C for 15 minutes, followed by 70°C in 5 minutes. The reaction was processed in ThermalCycler™ PCR (Eppendorf, Germany). 2 µl aliquots of each samples was made and stored at -20°C.

3.6.2. Reverse Transcriptase (RT)-PCR of *Anopheles* β-Actin

Anopheles β-actin primer AF: 5'- ATG TAC GTC GCC ATC CAG GC -3 ' and β-actin AR; 5'- CGA TGG TGA TGA CCT GTC CGT -3' (Senthil Kumar *et al*., 2008) was used as a house keeping gene for quantitative standardization of the cDNA samples. 25 µl PCR reactants included *Taq* polymerase buffer (1 X) (Annexture II), $MgCl_2$ (1.5 mM), dNTPs (0.25mM), primer (0.1pM each), *Taq* polymerase (1 U). The volume was made to 25 µl with DEPC treated water and different concentration of cDNA template. To obtain similar banding intensity, different concentrations of cDNA was made and PCR was repeated several times until a uniform banding pattern was obtained to ensure equal concentration of cDNA template. PCR condition consisted of initial denaturation at 94^{o}C for 1 minute, followed by 35 cycles of 94^{o}C for 30 seconds, 50^{o}C for 30 seconds of primer annealing, 72^{o}C for 30 seconds as primer extension and final extension at 72^{o}C for 1 minute.

Segment	Step	Temperature (^{o}C)	Times	Number of cycles
1	Initial denaturing	94	1 minute	1
2	Denaturing Annealing Extension	94 50 72	30 seconds 30 seconds 30 seconds	35
3	Final extension	72	1 minute	1

Table 1. Temperature cycling parameters for RT-PCR of *Anopheles* β-Actin

3.6.3. Study the expression of *Anopheles* Glutathione-S-Transferase epsilon-4 gene

Primers (AGSTe4F 5'- TAC ACG GCC AAA CTC AGC -3' and AGSTe4R 5'- CGG TAC AGA TTG TCG ATC -3') to obtained the partial expression of *Anopheles* GSTe4 gene was designed from NCBI database. 25 µl PCR reaction included *Taq* polymerase buffer (1X), $MgCl_2$ (1.5 mM), dNTPs (0.25mM), primer (0.1pM each), *Taq* polymerase (0.5 U) and cDNA template. The volume was made to 25µl with DEPC water. The concentration of cDNA template used for PCR was referred from standardized β-actin PCR result. PCR condition consisted of initial denaturation at 94^{o}C for 1 minute, followed by 35 cycles of 94^{o}C for 30 seconds, 58^{o}C for 30 seconds for primer annealing, 72^{o}C for 30 seconds as primer extension and final extension at 72^{o}C for 1 minute (Senthil Kumar *et al*., 2008).

Segment	Step	Temperature (^{o}C)	Times	Number of cycles
1	Initial denaturing	94	1 minute	1
2	Denaturing Annealing Extension	94 58 72	30 seconds 30 seconds 30 seconds	35
3	Final extension	72	1 minute	1

Table 2. Temperature cycling parameters for expression of *Anopheles* GSTepsilon-4 gene

GSTe4F ⟶

```
diruse4      ATGCCGAACATCAAGCTGTACACGGCCAAACTCAGCCCTCCGGGACGAGCGGTGGAGCTG 60
gambiaeE4    ATGCCAAACATTAAGCTGTACACGGCCAAACTCAGCCCACCGGGCCGGTCGGTCGAGCTG 60
                ***** ***** ************************** ***** **  **** ******

diruse4      ACGGGGAAGGCGCTGGGACTGGAGTTCGACATCTCCCCGATCAATCTGATCGCCGGAGAT 120
gambiaeE4    ACAGCAAAGGCGCTCGGGCTGGAGCTCGACATCGTGCCGATCAATCTGCTCGCGCAGGAA 120
                ** *  ******** ** ****** ********   ************ ****    **

diruse4      CACCTGCGGGAGGAGTTCCGGAAGCTGAATCCTCAGCACACGATCCCGCTGATCGACGAC 180
gambiaeE4    CATCTGACGGAAGCGTTCCGGAAGCTGAACCCGCAGCACACCATCCCGCTGATCGACGAC 180
                ** ***  *** * *************** ** ******** ******************

diruse4      GCCGGTACGATCGTGTACGAAAGCCACGCGATCATCGTGTACTTGGTGACGAAGTACGGC 240
gambiaeE4    AACGGGACGATCGTGTGGGACAGCCACGCCATCAATGTGTATCTGGTGAGCAAGTACGGC 240
                  *** **********  ** ******** ****  *****  ******  *********

diruse4      ------GCGGACGATAGCCTCTATCCGTCGGACGCGGTGACGCGCTCCAAGGTCAACGCG 294
gambiaeE4    AAGCCCGAGGGCGACAGTTTGTATCCGTCGGATGTGGTGCAACGGGCGAAGGTTAACGCG 300
                      * ** *** **  * *********** * ****   **  * ***** ******

diruse4      GCGCTACACTTCGATTCGGGTGTTCTGTTCGCCCGGCTGCGATTCTATTTGGAACCAATT 354
gambiaeE4    GCGCTACACTTCGATTCGGGCGTTCTGTTTGCCCGGTTCCGGTTCTATTTGGAACCAATA 360
                ******************** ******** ****** * ** *****************

                                                  <-------- GSTe4R
diruse4      CTGTACTACGGATCGACCGAAACACCGCAGGAGAAGATCGACAATCTGTACCGGGCGTAC 414
gambiaeE4    CTGTACTACGGAGCGACCGAGACACCGCAGGAAAAGATCGACAATCTGTACCGCGCGTAC 420
                ************ ******* *********** ******************** ******

diruse4      GAGCTGCTGAACGCCACGCTGGTCGACGATTACATCGTGGGAAGCCGGTTGACGCTGGCC 474
gambiaeE4    GAGCTGCTGAATGACACGCTGGTCGACGAGTACATCGTGGGCAACGAGATGACACTGGCC 480
                *********** * *************** *********** * *  * **** ******

diruse4      GATCTGAGCTGTGTTGCAAGCATCGCCTCGATGCATGCCATCTTCCCGATCGATGCCGGC 534
gambiaeE4    GATCTGAGCTGCATCGCCAGCATTGCTTCGATGCATGCGATTTTCCCGATCGATGCCGGC 540
                ***********  * ** ***** ** *********** ** ******************

diruse4      AAGTATCCGAAGCTGTTGGCCTGGGTCGAGCGTATCGCGAAGTTGCCCTACTATGCGGCG 594
gambiaeE4    AAGTATCCGAGGCTGGCCGGTTGGGTCAAACGCCTTGCCAAGCTGCCGTACTACGAGGCA 600
                ********** ****   *  ****** * **  * ** *** **** ***** * ***

diruse4      ACGAATCAGGCCGGTGCGGAAGAACTGGCCCAGCTGTATCACGCCAAGCTAGCGGAGAAC 654
gambiaeE4    ACGAATCGGGCCGGTGCGGAAGAGCTCGCTCAGCTGTACCGTGCCAAGTTGGAGCAAAAC 660
                ******* *************** ** ** ******** *  ****** * * * * ***

diruse4      CGTGCTAAAGCAAAGTGA 672
gambiaeE4    CGCACCAACGCCAAGTGA 678
                **  * ** ** ******
```

Figure 5. CLUSTAL W alignment of GSTe4 complete coding sequences of *Anopheles gambiae* (GenBank Accession NO. AY070254.1) and *Aedes aegypti* (GenBank Accession NO. AY819709.1). The highlighted region show the sequences selected for GSTe4 primer.

3.7. *In-silico* characterization of GST epsilon-4 of mosquito

Computational packages and online servers characterization GST epsilon-4 of mosquito proteins was done as the method described by Sivakumar *et al*. (2007). GSTe4 protein sequences of mosquito were retrieved from National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). NCBI is scanned for the key word Glutathione-S-transferase (GSTe4) of mosquito. From the search result yielded, 8 protein sequences of GSTe4 of mosquito were selected (*i.e.* for each Gluthione-S-transferase a protein sequence was chosen for each types of mosquito) by longest amino acids composition and have organized a non-redundant data set. The protein sequences of GSTe4 of mosquito were retrieved in FASTA format and used for analysis.

The amino acid composition of immune peptides sequences were computed using Expasy's ProtParam (http://us.expasy.org/tools/protparam.html) prediction server. Percentages of hydrophobic and hydrophilic residues were calculated from the primary structure analysis results using protprop software (http://www.mzu.edu.in/schools/biotechnology.html) and tabulated. The physico-chemical parameters, theoretical isoelectric point (pI), molecular weight, total number of positive and negative residues, extinction coefficient, half-life, instability index, aliphatic index and grand average hydrophathy (GRAVY) were computed using the Expasy's ProtParam prediction server and tabulated in table. The secondary structure and percentage of residues forming alpha, beta, and coil structures were predicted by a tool - Secondary Structural Content Prediction (SSCP) server (http://coot.embl.de/SSCP//sscp_seq.html). Percentages of hydrophobic and hydrophilic residues were calculated from the primary structure analysis

results using Protprop software (http://www.mzu.edu.in/schools/biotechnology.html) developed by Department of Biotechnology, Mizoram University.

The presence of disulphide bridges (SS bonds) in EAA07591.6, EAT42685.1 and EDS36584.1 were predicted by two methods. The first method involved the prediction of SS bonds using the primary structure (protein sequence data) by the tool CYS_REC (http://sun1.softberry.com/berry.phtml?topic= cys_rec&group=help &subgroup=propt.). CYS_ REC identified the positions and total number of cysteines present and predicted the most probable SS bond pattern of pairs (based on the matrix of pair scores) in the submitted FASTA format protein sequence. The second method involved the visualization and identification of SS bonds using the three-dimensional structure of protein (3D co-ordinates data). The protein sequences were submitted in EsyPred3D Webserver 1.0 (http://www.unamur.be/sciences/biologie/urbm/bioinfo/esypred/), a 3D model of the submitted protein was builded by the server and provided a protein data bank (PDB) file. The tool Rasmol (http://openrasmol.org/) was used to visualize the modelled 3D structures and to identify the SS bonds. The modelled 3D structures were evaluated using the Protein Quality predictor (ProQ)online server (http://www.sbc.su.se/~bjornw/ProQ/ProQ.html).

V. RESULTS

IV. RESULTS

4.1. Diversity, distribution and population studies of mosquito

4.1.1. General and common morphological features of a mosquito

- **Larva:** The larvae mostly float close to the water surface or to the margins of water. Mainly breathe by spiracles on 8th abdominal segment.
- **Adult**: Identified by their typical resting position: males and females rest with their abdomen sticking up in the air (60 degree) rather than parallel to the surface on which they are resting as well as maxillary palp as parallel to the proboscis.

Sub-genus – *Anopheles*: Wing with 3 or less than three dark spots on costa (1, 2, 3), which also involved vein 1.

Sub-genus – Cellia: Wings are with pale markings and has a presence of 4 or more dark spot on costa (1, 2, 3 and 4) which also involve vein 1.

General and common morphological features of Sub-family Culicinae

Larva: The larvae mostly hanging on the water surface (45^{o} to the water surface), mainly breath on siphonal tube at the base of the abdomen. The dorsal and ventral abdominal setae arise separately and usually without basal sclerites and mouth brushes are composed of numerous, usually slender filaments.

Adult: Identified by their typical resting position: males and females rest with their abdomens parallel to the surface on which they are resting. Maxillary palpi that is usually much shorter than the proboscis. Male have long palpi with numerous long setae, but they are not swollen apically like those of anophelines. The wing veins are entirely dark-scaled, but speckles or patches of white or yellow scales are present in some species.

Sub-genus – *Culex*: They are usually drab, unicolorous mosquito, but some species of subgenus *Culex* have markings on the legs and pale spot on the wings similar to *Anopheles*. They are characterized by the presence of distinct pulvilli (one of the two pad-like or devided lobes on the post-tarsus) and the absence of pre-spiracular setae and of post-spiracular setae (setae occurring on the spiracular area). Abdomen usually blunt.

Sub-genus – *Culiseta*: They are rather large mosquito which bears a superficial resemblance to *Culex*. They lack distinct pulvilli (one of the two pad-like or devided lobes on the post-tarsus) and posses pre-spiracular setae and of post-spiracular setae (setae occurring on the spiracular area) and bear a patches of setae at the base of the subcosta on their ventral surface of the wing.

Sub-genus – *Aedes*: They are relatively small mosquito. They are characterized by large pale bands or tarsi ringed with white bands. Abdominal bands with v-shaped notch and are pointed. Thorax with pale bands that extend to the head. Wings with entirely dark scales.

Sub-genus – *Toxorhynchites*: They are the largest mosquito and are easily recognized by their large size and strongly bent proboscis. The body is covered with brightly coloured iridescent scales and the posterior abdominal segments have lateral scale-tufts. Larvae are very large and easily recognized. They vary from pink to red and purple in colour.

Species	Palpi	Wings	Legs
Anopheles barbirostris	entirely dark scales	fringe spot at vein 5.2., 3rd and 5th wing vein	hindfemur entirely dark, tuft of scales on 8th abdominal segment
Anopheles vagus	pre-apical dark on palpi	fringe spot present on all veins	apical pale bands on joints of tarsomere
Anopheles philippinensis	apical pale band is nearly equal to the pre-apical dark band	Cubitus largely pale scaled, presector dark mark on vein 1	hind legs tarsomere 5, 4 and 3 are completely white
Anopheles jamesii	apical and sub-apical pale band are nearly equal	At least 4 dark areas involving both costa and vein R; vein 5.1 pale at base	femura and tibiae speckled; tarsomeres 5, 4 and 3 are completely white
Anopheles jeyporiensis	sub-apical dark band is much broader; tip is pale	Pale spot on vein 1st and 3rd	fore tarsi with narrow pale bands
Anopheles dirus	fore banded	4 dark areas involving both costa and vein R_1; presector dark mark vein 1	presence of a big pale band between 1st and 2nd hindtarsi
Anopheles minimus	apical and sub-apical pale bands broader than sub-apical dark band	vein 3 mainly white; inner costa with one pale interruption; vein 5.1 is with three dark spots	tarsomeres 1-4 are with very small dorso-apical pale patches.
Anopheles nivipes	apical pale band in nearly equal to the pre-apical dark band	presector dark mark on vein 1; Wing vein 5 mainly white no dark spot	tarsomeres 5, 4 and 3 are completely white
Anopheles willmori	palpi with or without pale spots on segment 3	4 or more dark spots on costa (1, 2, 3 and 4) which also involve vein 1.	Speckled legs; hind tarsomere 5 white

Table 3. Main identification characters of collected Anopheline mosquitoes.

Species	Proboscis	Wings	Legs	Abdomen
Culex quinquefasciatus	entirely dark scales	scales entirely dark	tarsi not ringed with white band	round pale band at the base of the abdomen
Culex tritaeniorhynchus	large pale band at the pre-apical region	dark scales	hind femur pale; narrow dark ring dist	abdominal terga with narrower basal pale bands
Culex bitaeniorhyunchus	pre-apical pale band larger than apical dark band	heavily speckled with pale band on the veins	Femur and tibia with speckled	abdominal terga II-VII with evenly broad apical pale band
Culex peus	Pre-apical pale band	Wings entirely dark	pale line on the underside of femur	-
Culex tarsalis	Pre-apical pale band	pale bands on sub-costa region	under side of the hind femur pale	-
Culex mimeticus	Pre-apical pale band	spotted with three white spots on the costa	tarsal joint is completely white	-
Aedes albopictus	entirely dark	entirely dark	hindtarsomere with large pale bands; 5^{th} entirely white	pale spots, longitudinal white stripe on thorax
Aedes aegypti	entirely dark	entirely dark	Femur with white knee-spot	pale spots. sub-median narrow longitudinal stripe on thorax
Culiseta melanura	entirely dark	entirely dark	scattered pale scales	basal bands
Culiseta inornata	entirely dark	pale scales present on costa, subcosta, and radius	scattered pale scales	entirely dark-scaled but may have basal bands
Toxorhynchites splenden.	strongly bent downward; metallic reflection	entirely dark	purple metallic reflection on hind tarsi	metallic blue-green scales dorsally

Table 4. Main identification characters of collected Culicidae mosquitoes.

4.1.1.1. Key features for adult mosquito species - *Anopheles*

Anopheles barbirostris

Author: Van der Wulp, 1884

Morphological indentification characters:

Palpi: Entirely dark scales without any pale marking

Wings: Inner quarter of costa with scattered pale scales, usually with a fringe spot at vein 5.2. Presence of fringe spot at the end of the 3rd and 5th wing vein. basal half of anal vein with scattered dark scales.

Legs: Hind femur without a broad white band.

Abdomen: Female with a prominent tuft of scales on ventral surface of abdominal segment VII.

Anopheles vagus

Author: Doenitz, 1902

Morphological identification characters:

Palpi: Maxillary palpus with a pre-apical dark band not more than half the length of apical pale band

Wings: Fringe spot present on all veins, it was also observed between vein 5 and 6 and also beyond vein 6.

Legs: Joints between fore tarsomeres with broad basal and apical pale bands. Femur and tibia not speckled usually dark in the hind legs, hind tarsomere 5 dark scaled.

Anopheles philippinensis

Author: Ludlow, 1902

Morphological identification characters:

Palpi: The apical pale band is nearly equal to the pre-apical dark band.

Wings: Cubitus largely pale scaled, without any dark area, presectoral dark mark on vein 1 shorter, either of same length as on costa or extending basally beyond the end of corresponding dark mark on costa, but not reaching the distal end of humeral dark mark on costa.

Legs: Femur and tibia not speckled, hind legs tarsomere 5, 4 and 3 are completely white, joint between tarsomere 1 usually with white band.

Anopheles jamesii

Author: Theobald, 1901

Morphological identification characters:

Palpi: Apical and sub-apical pale band are nearly equal.

Wings: At least 4 dark areas involving both costa and vein R; inner quarter and outer third of costa chiefly pale; vein 5.1 pale at base

Legs: Femura and tibiae speckled. Hind tarsi with at least two apical tarsomeres are completely white and tarsomeres 5, 4 and 3 are completely white.

Abdomen: Dorsum of VII and VIII abdominal segments covered with golden scales.

Anopheles jeyporiensis

Author: James, 1902

Morphological identification characters:

Palpi: Female palpi with subapical pale band are narrow and the sub-apical dark band is much broader; tip is pale.

Wings: Basal third of costa with one or more white interruptions; vein 3 mainly pale; pale spot present on other wing veins besides costa and 1st vein.

Legs: Femura and tibiae not speckled; hind tarsomere 5 is not white and fore tarsi with narrow pale bands.

Anopheles dirus

Author: James, 1902

Morphological identification characters:

Palpi: The palpi are for banded.

Wings: At least 4 dark areas involving both costa and vein R_1; presectoral dark mark vein 1 with one or more pale interruptions.

Legs: Femur and tibia speckled with the presence of a big pale band without a ventral dark stripe is observed at its junction.

Anopheles minimus

Author: Theobald, 1901

Morphological identification characters:

Palpi: Pale at tip; the palpi with both apical and sub-apical pale bands as broad as or broader than sub-apical dark band.

Wings: Wing vein 3 mainly white; inner costa with one pale interruption; vein 5.1 is with three dark spots; vein 2.1dark except at base and apex.

Legs: Fore tarsi are unbanded and the tarsomeres 1-4 are with very small dorso-apical pale patches.

Anopheles nivipes

Author: Theobald, 1903

Morphological identification characters:

Palpi: The apical pale band in nearly equal to the pre-apical dark band.

Wings: Presectoral dark mark on vein 1 long, usually reaching or overlapping the distal end of the humeral dark mark on costa. Wing vein 5 mainly white, with no dark spot at the point of bifurcation.

Legs: Femur and tibia not speckled; hind legs and tarsomeres 5, 4 and 3 are completely white; apex of hind tarsomere 1 usually with white band.

Anopheles willmori

Author:Theobald, 1903

Morphological identification characters:

Palpi: Maxillary palpus with a pre-apical dark band not more than half the length of apical pale band; palpi with or without pale spots on segment 3.

Wings: Wings with 4 or more dark spots on costa (1, 2, 3 and 4) which also involve vein 1.

Legs: Femur and tibia speckled. Hind tarsi with less than two apical tarsomeres completely white (hind tarsomere 5 white).

4.1.1.2. Key features for adult mosquito species - *Culex*

Culex quinquefasciatus

Author: Theobald, 1903

Morphological identification characters:

Palpi: The palpi much shorter than proboscis; proboscis entirely dark scales.

Wings: Wing clear; average size; wing scales entirely dark.

Legs: Tarsi not ringed with white band.

Abdomen: Bands on the base of each abdominal segment; round pale band at the base of the abdomen which is like half Moon shaped.

Culex tritaeniorhynchus

Author: Giles, 1901

Morphological identification characters:

Proboscis: Large pale band at the pre-apical region; Proboscis usually with accessory pale patches or stripe on ventral surface.

Wings: Entirely dark scales and the absence of pale marking.

Legs: Hind femur pale with distinct, narrow dark ring distally, tarsomere entirely dark scales.

Abdomen: Abdominal terga with narrower basal pale bands, usually smaller size.

Culex bitaeniorhynchus

Author: Giles, 1901

Morphological identification characters:

Proboscis: Pre-apical pale band larger than apical dark band.

Wings: Heavily speckled with pale band on the veins.

Legs: Femur and tibia with speckled; hind tarsomere entirely dark scales with not pale band at the joints of the tarsomeres.

Abdomen: Abdominal terga II-VII with evenly broad apical pale band without api-colateral pale patches.

Culex peus

Author: Speiser, 1979

Morphological identification characters:

Proboscis: Pale band on proboscis.

Wings: Entirely dark.

Legs: Femur and tibia with not speckled; hind tarsomere entirely dark scales with narrow pale line on the underside of hind femur.

Culex tarsalis

Author: Coquillett, 1896

Morphological identification characters:

Proboscis: Pre-apical pale band equal or nearly equal to the apical dark band.

Wings: At least two pale bands on sub-costa region. wing's vein with entirely dark.

Legs: Femur and tibia with not speckled; hind tarsomere entirely dark scales with under side of the hind femur pale.

Culex mimeticus

Author: Teixeirain, 1909

Morphological identification characters:

Proboscis: Pre-apical pale band equal or nearly equal to the apical dark band.

Wings: Wings spotted with three white spots on the costa. The first longitudinal vein has two black bands and the tip black.

Legs: Femur a uniform light yellow, the tibia is light brown with a pale knee spot. The meta-tarsus and first two tarsal joints have apical and basal bands of white; the last tarsal joint is completely white.

4.1.1.3. Key features for adult mosquito species - *Aedes*

Aedes albopictus

Author: Skuse, 1894

Morphological identification characters:

Proboscis: Proboscis without white band.

Wings: Entirely dark.

Legs: Femur with white knee-spot; mid-femur without longitudinal white patches on anterior surface; hind tarsomere with large pale bands; hindtarsomere 5 entirely white.

Thorax: Scutum with a narrow median longitudinal white stripe

Aedes aegypti

Author: Linnaeus, 1762

Morphological identification characters:

Proboscis: Proboscis without white band.

Wings: Entirely dark.

Legs: Femur with white knee-spot; mid-femur with longitudinal white patches on anterior surface; hind tarsomere with large pale bands; hind tarsomere 5 entirely white.

Thorax: Scutum black or brown with a pair of sub-median narrow longitudinal white stripe.

4.1.1.4. Key features for adult mosquito species - *Culiseta*

Culiseta melanura

Author: Coquillett, 1902

Morphological identification characters:

Proboscis: Proboscis without white band.

Wings: Scattered entirely dark; average size.

Legs: Tarsomeres entirely dark-scales or with scattered pale scales.

Abdomen: Abdomen usually entirely dark-scaled but may have basal bands.

Culiseta inornata

Author: Williston, 1893

Morphological identification characters:

Proboscis: Proboscis without white band.

Wings: Scattered pale scales present on costa, subcosta, and radius.

Legs: Tarsi with many, scattered pale scales

Abdomen: Abdomen usually entirely dark-scaled but may have basal bands.

4.1.1.5. Key features for adult mosquito species - *Toxorhynchites*

Toxorhynchites splenden

Author: Theobald, 1901

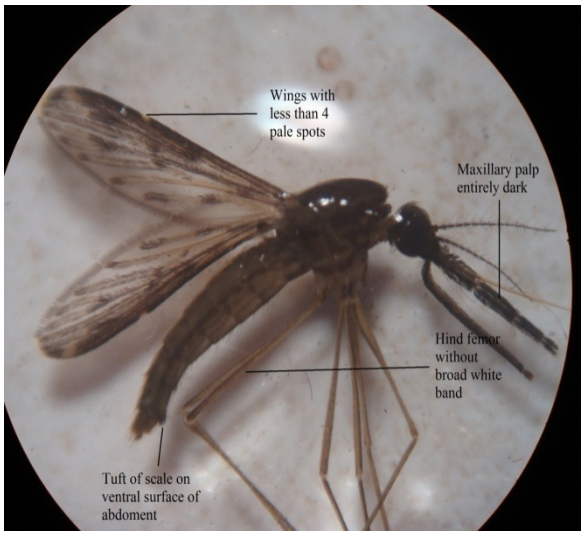
Morphological identification characters:

Proboscis: Proboscis entirely dark; strongly bent downward; dark scales of palpus with metallic reflections.

Wings: Entirely dark.

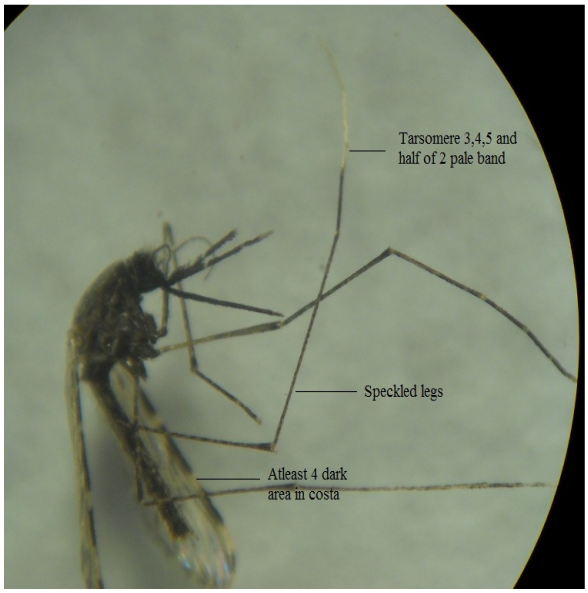
Legs: Hind tarsi with purple metallic reflections.

Abdomen: Abdomen with metallic blue-green scales dorsally and yellow scales laterally and ventrally.



6A. ***Anopheles barbirostris***

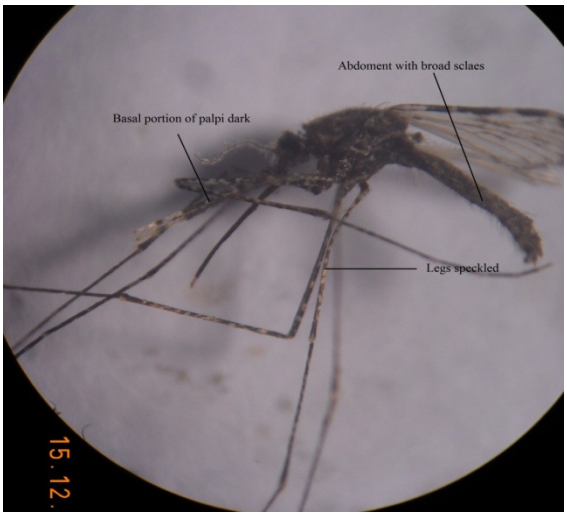
6B. ***Anopheles jamesii***



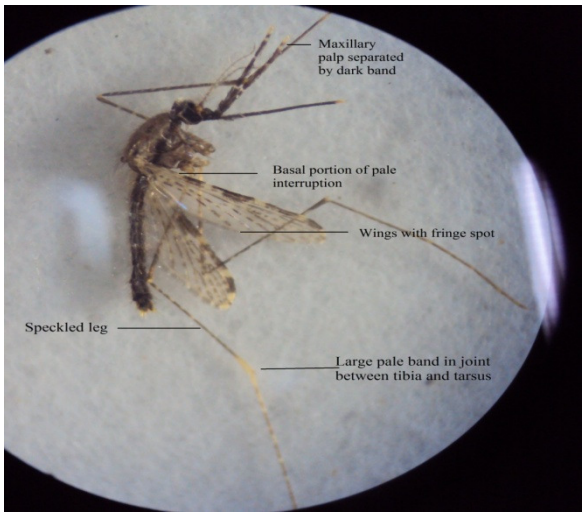
6C. ***Anopheles philipinensis***

6D. ***Anopheles vagus***

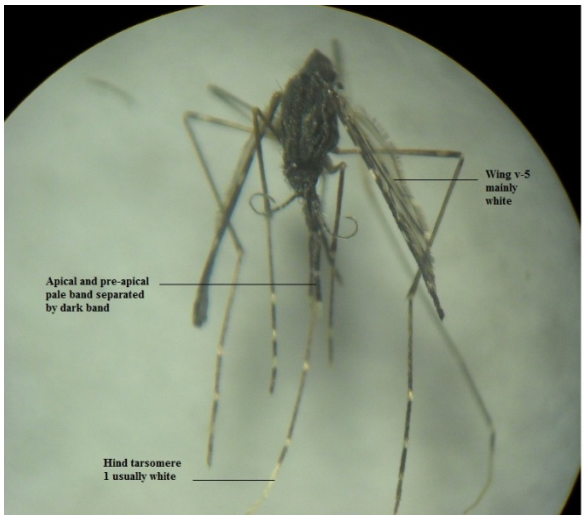
Figure 6A-D. Collection of adult *Anopheles* mosquito species with their main identification charecters.



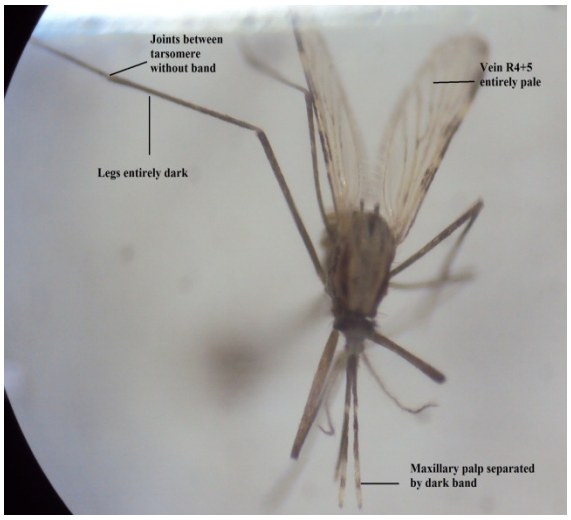
7A. ***Anopheles willmori***



7B. ***Anopheles dirus***



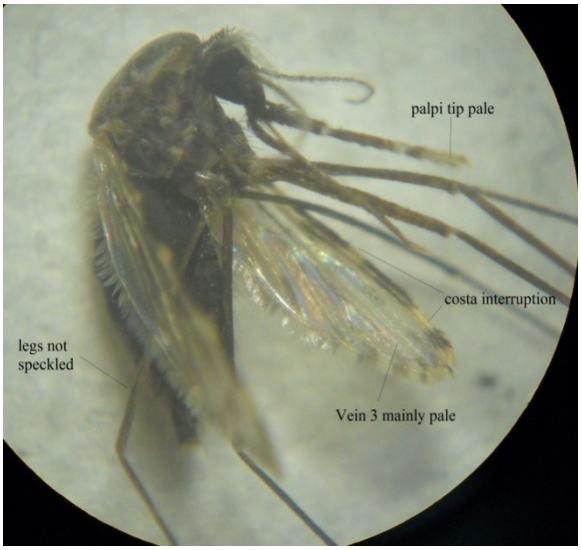
7C. ***Anopheles nivipes***



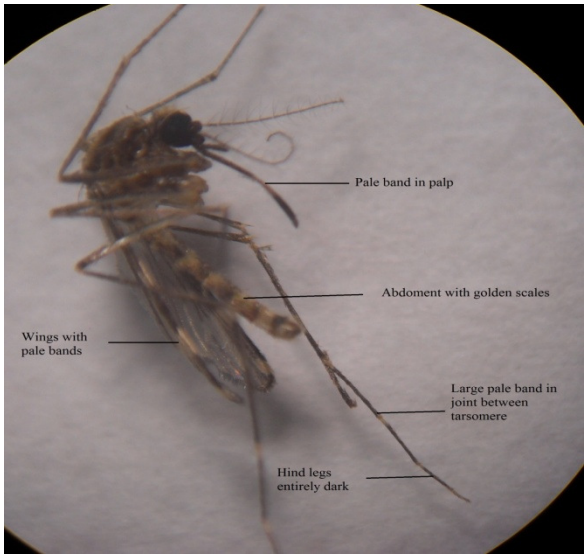
7D. ***Anopheles minimus***

Figure 7A-D. Collection of adult *Anopheles* mosquito species with their main identification charecters.

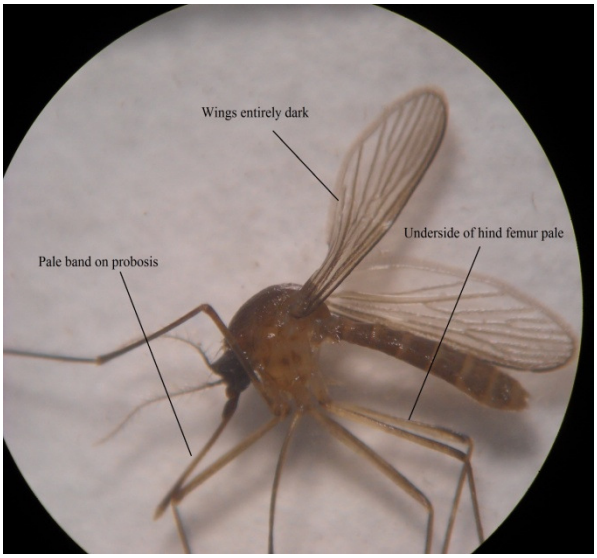
8A. ***Culex quinquefasciatus***



8B. ***Anopheles jeyporiensis***

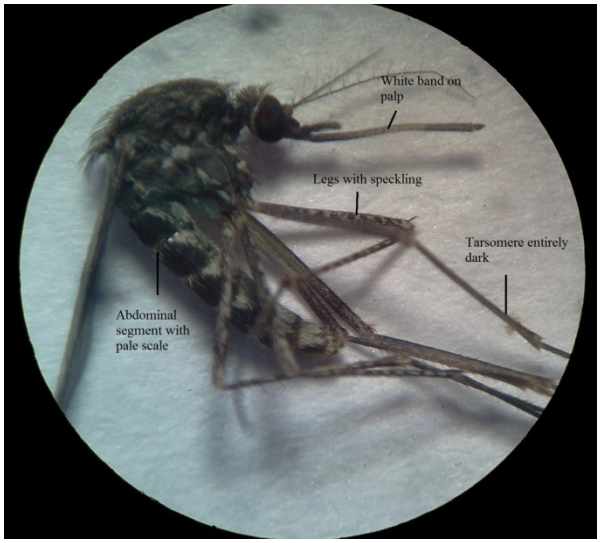


8C. ***Culex mimeticus***

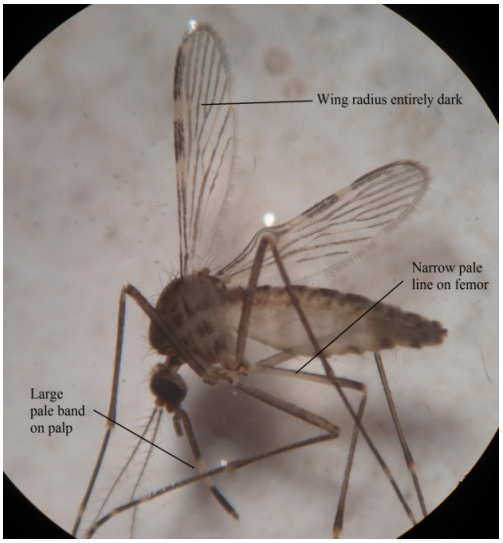


8D. ***Culex peus***

Figure 8A-D. Collection of adult *Anopheles* and *Culex* mosquito species with their main identification charecters.

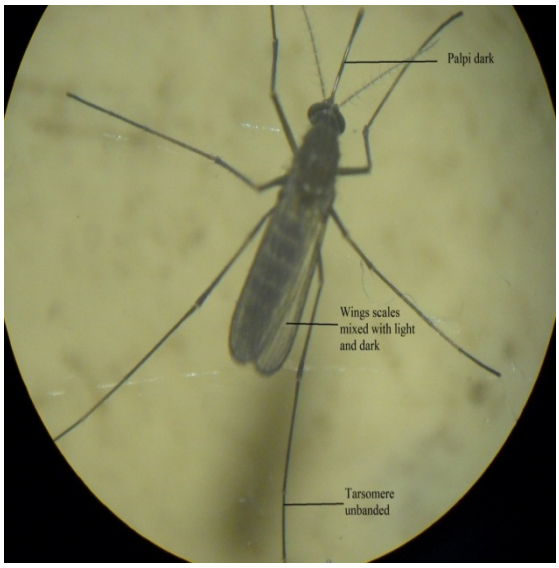


9A. *Culex bitaeniorhynchus*



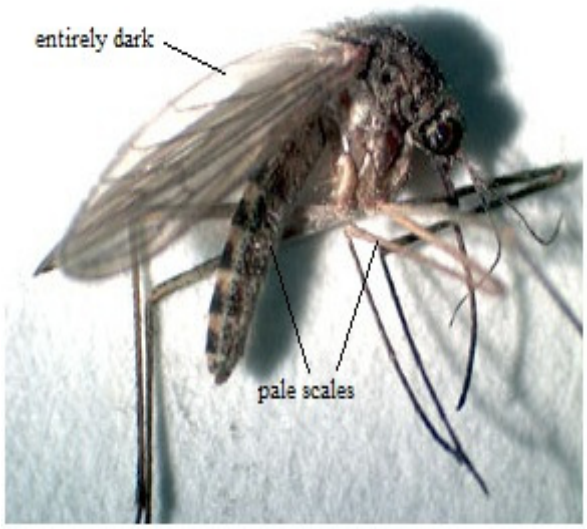
9B. *Culex tarsalis*

9C. *Culex tritaeniorhynchus*

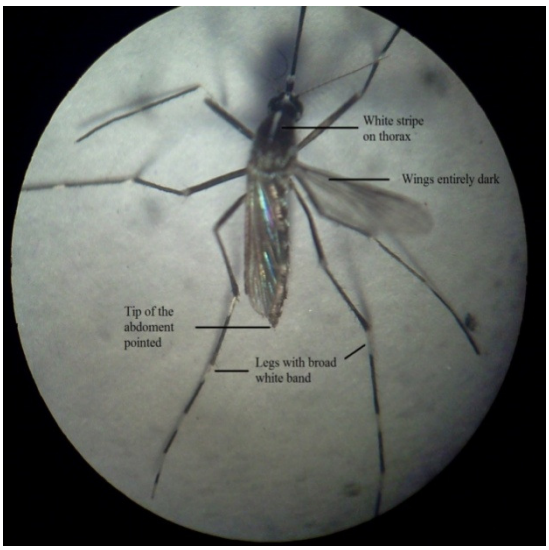


9D. *Culiseta melanura*

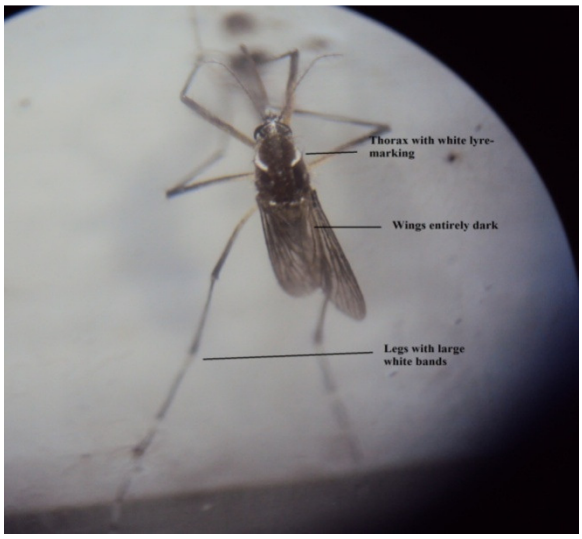
Figure 9A-D. Collection of adult *Culex* and *Culiseta* mosquito species with their main identification charecters.



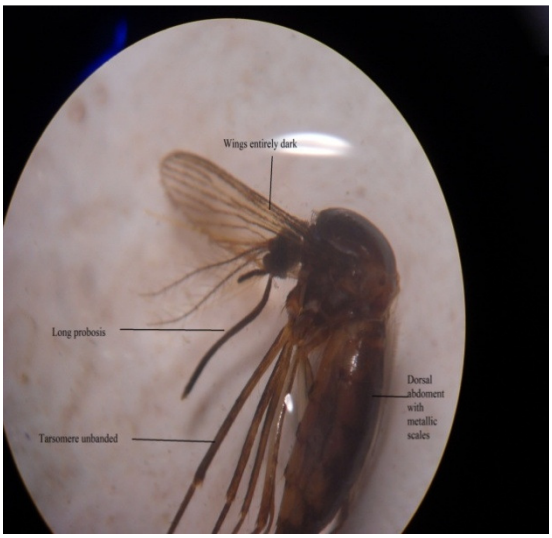
10A. ***Culiseta inornata***



10B. ***Aedes albopictus***



10C. ***Aedes aegypti***



10D. ***Toxorhynchites splenden***

Figure 10A-D. Collection of adult *Aedes, Culiseta* and *Toxorhynchites* mosquito species with their main identification charecters.

Species	Mode of collection	L/A	Aizawl	Kolasib	Serchhip	Mamit	Lunglei	Lawngtlai
Cxm	SN	L	231	-	-	-	-	-
Cum	SN	L	14	53	-	-	-	-
Txr	SN	L	28	17	-	-	9	-
Cxt	SN	L	85	-	-	-	-	-
Cxu	SN	L	68	-	-	-	-	-
Cxq	SN,HC	L,A	1358	1178	460	88	153	66
Cxr	SN	L	253	-	-	-	-	-
Cxb	SN	L	162	-	-	-	-	-
Aea	SN,HC	L,A	199	-	45	5	23	28
And	LT	A	-	-	-	2	1	-
Anb	SN,LT	L,A	1447	443	254	75	94	132
Cui	SN	L	-	28	-	-	-	-
Anv	SN,LT	L,A	1057	45	50	45	6	5
Anj	SN	L	-	2	2	-	5	-
Ani	SN,HC	L,A	12	-	8	4	-	-
Aey	SN	L	-	-	-	2	-	-
Ann	SN,HC	L,A	-	-	12	-	4	-
Anm	SN,HC	L,A	-	-	4	2	-	-
Anp	SN,LT	L,A	28	6	2	4	-	-
Anw	SN	L	6	-	-	6	-	-

Table 5. Distribution and population size of mosquito across six districts of Mizoram.

SN – Scoup-net, LT – Light trap, HC – Hand Collection. L – Larvae, A – Adult. *Anb – Anopheles barbirostris, Anv- Anopheles vagus, Anj- Anopheles jeyporiensis, Ani- Anopheles jamesii, Aey– Aedes aegypty, Aea – Aedes albopictus, Cum- Culiseta Melanura, Cui – Culiseta Inornata, Cxq – Culex quinquefasciatus, Cxt – Culex tarsalis, Cxu – Culex peus, Cxr- Culex tritaerniorhynchus, Cxm- Culex mimeticus; Cxb- Culex bitaerniorhynchus, Anm- Anopheles minimus, Ann- Anopheles nivipes, Anw- Anopheles willmori, Anp- Anopheles philipinensis. Txr- Toxorhynchites splenden.*

4.1.2. Survey of mosquito population in six districts of Mizoram

Mosquito population survey was done in six districts of Mizoram using methods of collection described by WHO (1975). Adults were collected at dusk and midnight (4:00 – 8:00 pm; 12:00 – 2:00 am) using CDC light trap and hand collection while larvae were collected by scoup-net method (21 - 38^{o}C; 25 - 98% RH). The following were the collections of mosquito during 2009 to 2011 (Table 5).

Anopheles barbirostris was widely distributed across Mizoram. It was collected on all the survey six districts (54-1150m elevation) of Mizoram and dominant species among the Anopheline group. Larva and adults forms were collected during the study period (2009-2011). Larval forms were collected on ponds, river beds, rice fields and ditches while resting adults found in cattle sheds, both indoor and outdoor of human residence. Collection was done by means of scoup-net method as well as light trap. A total of 2455 species were collected during the study period: of which 1447 species from Aizawl, 443 species from Kolasib, 254 species from Serchhip, 75 species from Mamit, 94 species from Lunglei and 132 species from Lawngtlai.

Anopheles vagus was collected on all the surveyed six districts of Mizoram with the altitudinal variation of 54-830m. It was the second dominant species among Anopheline. Larva and adults forms were collected during the study period (2009-2011). Larval forms were collected on ponds, river beds, ditches with a clear water bodies while resting adults found in cattle sheds, both indoor and outdoor of human residence. Collection was done by means of scoup-net method as well as light trap. A total of 568 species were collected during study period:

of which 417 species from Aizawl in all study sites (Table 3) except Dinthar river, 45 species collected from Kolasib in Rubber board ponds, 50 species from Serchhip, 45 species from Mamit, 6 species from Lunglei and 5 species from Lawngtlai.

Anopheles philippinensis was collected from 4 districts (86-804m elevation) during the surveyed periods (2009-2011). Larval forms were collected on ponds, river beds and rock holes with thin vegetation while resting adults found in outdoor of human residence. Collection was done by means of scoup-net method as well as light trap and mosquito bat. In Lengpui, it was collected in pond with narrow vegetation and slightly turbid but the rest of the species were collected in small habitats with a clear water bodies. A total of 40 species collected; of which 28 species from Aizawl in cattle shed near Lengpui fish pond and Mission veng River, 6 species from Kolasib, 2 species from Serchhip and 4 species from Mamit in Bawngva pond.

Anopheles jamesii was collected from 3 districts during the study periods. Larval forms were collected by scoup-net method in ponds, river beds with slow flowing. Adults were collected by means of hand collection in outdoor (cattle shed). A total of 24 species collected during the study period; of which 12 species from Aizawl in fish pond (405m), 8 species from Serchhip in river slow stream (804m) and 4 species from Mamit in bawngva pond (86m).

Anopheles jeyporiensis was collected from 3 districts during the study period in larval forms by means of scoup-net method. It was collected from stream and river edge where there is less water current. A total of 9 species were collected: of which 2 species from Kolasib in college veng pond (640m), 2 species from Serchhip in river beds near rice field (795m) and 5 species from Lunglei district in Chawngte rice field (86m).

Anopheles dirus a primary vector of malaria in Mizoram (Das *et al*., 1990) was collected in few numbers. Collection was done by means of light trap in cattle shed and outdoor of human dwelling. A total of 3 species collected during the study period: of which 2 species collected from Mamit in Bawngva village of outdoor near human dwelling (86m) and 1 species from Lunglei in chawngte village near base camp of labourers (80m).

Anopheles minimus a vector of malaria in Mizoram (Das *et al*., 1990) was collected in two districts (75-804m) during the study period. Collection was mainly done by scoup-net method and hand collection. A total of 6 species collected; of which 4 species from Serchhip in rock holes near rice field and 2 adults species from Mamit in bawngva village.

Anopheles nivipes was collected in two districts (80-804m) during the study period. Collection was mainly done by scoup-net method and hand collection. A total of 16 species collection: of which 12 species were collected from Serchhip in rock holes and 4 species from Lunglei in chawngte village.

Anopheles willmori collection was done by scoup-net method as only the larval forms were collected. A total of 12 species collected: of which 6 species from Aizawl in Dinthar River (river edge) with less water current (912m) and 6 species from Mamit in Bawngva and Darlak fish pond (75-80m).

Culex quinquefasciatus a dominant species and vector of filariasis was found throughout the entire six districts of Mizoram during the study period. Collection was done by scoup-net method for larvae, hand collection and mosquito bat for the adults. It was found in all the study sites (75-1115m) wherein out of 3303 total species collected: 1358 species from Aizawl, 1178

species from Kolasib, 460 species from Serchhip, 88 species from Mamit, 153 species from Lunglei and 66 species from Lawngtlai.

Culex tritaeniorhynchus a vector of Japanese encephalitis (Kanoja, 2007) was found in Aizawl district only. Collection was mainly done by scoup-net method as only the larval forms were collected. A total of 253 species collected: of which 245 species were collected from Lengpui fish pond (405 m) and Ramrikawn ditches (871m).

Culex bitaeniorhyunchus reported to be naturally infected with Japanese encephalitis virus (Kanoja, 2007) was collected from Aizawl district during the study period. Larval forma were collected by scoup-net method. Out of 162 total species collected: of which 156 species from Lengpui pond in Aizawl (405m) and 6 species from Ramrikawn ditches (871m).

Culex peus was found only in Ramrikawn ditches (871.42m) of Aizawl district. Collection was done by means of scoup-net method as only the larval forms were collected with a total of 68 species.

Culex tarsalis was also found only in Ramrikawn ditches of Aizawl district. A total of 85 species were collected by scoup-net method.

Culex mimeticus a rare species in Mizoram were collected from Lengpui fish pond (405m) and sihhmui semi-permanent pool (98m) of Aizawl district by scoup-net method. A total of 231 species were collected.

Aedes albopictus was found in four districts (98-1105m) of Mizoram. Collection was done by means of scoup-net method and hand collection. A total of 302 species collected: of which 206 species from Aizawl in Lengpui fish pond, ramrikawn water tank and sihhmui near semi-permanent pool in adult form; 45 species from Serchhip in pond, 23 and 28 species from Lunglei and Lawngtlai districts respectively.

Aedes aegypti was found in tree holes and tanks of Darlak villages (78m) near main road. A total of 2 species were collected from Mamit district.

Culiseta melanura was collected from Mision veng river (940m) of Aizawl district (14 species) and 53 species collected from Kolasib in college veng pond (640m) and Rengtekawn ditches (475m) by means of scoup-net method.

Culiseta inornata was found only in college veng pond (640m) of Kolasib district with a total collection of 28 species by means of scoup-net method.

Toxorhynchites splenden a predator of mosquito larvae was found in three districts (640-1105m) during the study period using scoup-net method. A total of 54 species collected; of which 24 species from mission veng river of Aizawl, 17 species from Kolasib in pond and ditches, 9 species from Lunglei park (water tank).

4.1.3. Diversity and distribution of Mosquito in Mizoram

A total of 8328 mosquitoes, representing the 5 genera of: *Anopheles*, *Aedes*, *Culiseta*, *Culex* and *Toxorhynchites* were collected altogether of 20 species (Table 6). They were found in a wide variety of habitats with the altitudinal variation of 54 - 1150m. The zones of lower elevation shared higher species abundance than the higher elevation. The most dominant genus was *Anopheles* followed by *Culex*, *Culiseta*, *Aedes* and *Toxorhynchites.* In the overall survey, the most dominant species was found to be *Culex quinquefasciatus* (39.67%) followed by *Anopheles barbirostris* (29.50%), *Anopheles vagus* (14.51%), *Aedes albopictus* (3.62%), *Culex tritaeniorhynchus* (3.03%), *Culex mimeticus* (2.78%), *Culex bitaeniorhyunchus* (1.94%), *Culex tarsalis* (1.02%), *Culiseta melanura* (0.80%), *Culex peus* (0.82%), *Toxorhynchites splenden* (0.64%), *Anopheles philipinensis* (0.48%), *Culiseta inornata* (0.33%), *Anopheles jamesi* (0.29%), *Anopheles nivipes* (0.19%), *Anopheles willmori* (0.14%), *Anopheles jeyporiensis* (0.10%), *Anopheles minimus* (0.07%), *Anopheles dirus* (0.03%) and *Aedes aegypty* (0.02%). Among these, 11 species were collected in immature forms, 8 species were collected in adult as well as immature while *Anopheles dirus* was collected only in adult form respectively (Table 7).

4.1.4. Abundance of mosquito species

Distribution and relative abundance studies (Table 8) from 18 survey sites in different districts of Mizoram suggested that only one species- *Culex quiquefasciatus* was mostly found throughout year and regarded as constant (C=80.1 - 100%). Two species (*Anopheles barbirostris* and *Anopheles vagus*) were frequent in most of the months (60.1 - 80%) while four species: *Aedes albopictus, Anopheles philipinensis, Anopheles jamesi, Toxorhynchites splenden.* were considered as infrequent (20.1 - 40%).

Collection sites	*Cxm*	*Cum*	*Txr*	*Cxt*	*Cxu*	*Cxq*	*Cxr*	*Cxb*	*Aea*	*And*	*Anb*	*Cui*	*Anv*	*Anj*	*Ani*	*Aey*	*Ann*	*Anm*	*Anp*	*Anw*
Aizawl District																				
Lengpui	213	-	-	-	-	856	245	156	140	-	1250	-	985	-	12	-	-	-	20	-
Dinthar	-	-	23	-	-	126	-	-	-	-	12	-	-	-	-	-	-	-	-	6
Sihhmui	18	-	-	-	-	248	8	6	56	-	185	-	53	-	-	-	-	-	-	-
Ramrikawn	-	-	-	85	68	128	-	-	5	-	-	-	6	-	-	-	-	-	-	-
Mission	-	14	5	-	-	-	-	-	-	-	-	-	13	-	-	-	-	-	8	-
sub total	231	14	28	85	68	1358	253	162	199		1447		1057		12				28	6
Kolasib District																				
Diakkawn	-	48	15	-	-	486	-	-	-	-	235	28	-	2	-	-	-	-	6	-
Rubber board	-	-	-	-	-	235	-	-	-	-	123	-	45	-	-	-	-	-	-	-
Rengtekawn	-	5	2	-	-	457	-	-	-	-	85	-	-	-	-	-	-	-	-	-
sub total		53	17			1178					443	28	45	2					6	
Serchhip District																				
Ponds	-	-	-	-	-	235	-	-	45	-	156	-	8	-	-	-	-	-	-	-
Rock holes	-	-	-	-	-	-	-	-	-	-	86	-	36	2	8	-	12	4	2	-
Rice field	-	-	-	-	-	225	-	-	-	-	12	-	6	-	-	-	-	-	-	-
sub total						460			45		254		50	2	8		12	4	2	
Mamit District																				
Lungsir	-	-	-	-	-	15	-	-	-	-	23	-	-	-	-	-	-	-	-	-
Bawngva	-	-	-	-	-	28	-	-	-	2	52	-	45	-	-	-	-	-	4	4
Darlak	-	-	-	-	-	45	-	-	5	-	-	-	-	-	4	2	-	2	-	2
sub total						88			5	2	75		45		4	2		2	4	6
Lunglei District																				
Lunglei park	-	-	9		-	18	-	-	23	-	12	-	-	-	-	-	-	-	-	-
Chawngte	-	-	-	-	-	135	-	-	-	1	82	-	6	5	-	-	4	-	-	-
sub total			9			153			23	1	94		6	5			4			
Lawngtlai District																				
Ponds	-	-	-	-	-	21	-	-	-	-	120	-	5	-	-	-	-	-	-	-
Tanks	-	-	-	-	-	45	-	-	28	-	12	-	-	-	-	-	-	-	-	-
sub total						66			28		132		5							
G.Total	231	67	54	85	68	3303	253	162	302	3	2455	28	1208	9	24	2	16	6	40	12

Table 6. Distribution and abundance of collected mosquitoes across six districts in Mizoram 2009-2011.

Name of the species	Phytogegraphic zones - District – Mizoram State					
	Aizawl 98-940m	Kolasib 54-640m	Mamit 74-850m	Lunglei 80-1150m	Lawngtlai 830-855m	Serchhip 804-961m
Culicidae: Culisinae: Culisetini: Culiseta						
Culiseta melanura	L	L				
Culiseta Inornata	L	L	L			
Culicidae : Culicinae : Culisini: Culex						
Culex mimeticus	L	L				
Culex quinquefasciatus	A,L	L	A,L	A,L	A,L	L
Culex tarsalis	L					
Culex peus	L					
Culex tritaeniorhynchus	L					
Culex bitaerniorhynchus	L					
Culicidae : Culicinae : Aedini: *Aedes*						
Aedes albopictus	A,L			A,L	A,L	
Aedes aegypti			L			
Culicidae:Anphelinae:Anphelini: Anopheles						
Anopheles willmori	L		L			
Anopheles nivipes				A,L		L
Anopheles philipinensis	A,L	L	A,L			L
Anopheles dirus			A	A		
Anopheles minumis			A			L
Anopheles barbirostris	A,L	A,L	A,L	L	L	A,L
Anopheles vagus	A,L	A,L	L	A,L	A,L	A,L
Anopheles jeyporiensis	L			L		L
Anopheles jamesii	A,L		L			L
Culicidae : Culicinae : Toxorhynchitini: Toxorhynchus						
Toxorhynchites splenden	L	L		L		

Table 7. Collection of mosquitoes across the six different districts of Mizoram (India) during 2009 to 2011. A - Presence of adult; L - Presence of larvae.

	Cxm	*Cum*	*Txr*	*Cxt*	*Cxu*	*Cxq*	*Cxr*	*Cxb*	*Aea*	*And*	*Anb*	*Cui*	*Anv*	*Anj*	*Ani*	*Aey*	*Ann*	*Anm*	*Anp*	*Anw*
Distribution %	11.11	16.67	27.78	5.55	5.55	**83.32**	11.01	11.11	33.33	11.11	77.78	11.11	61.12	16.67	22.22	5.56	11.11	11.11	22.21	16.67
Density %	2.77	0.80	0.65	1.02	0.82	**39.66**	3.04	1.95	3.63	0.04	29.48	0.34	14.51	0.11	0.29	0.02	0.19	0.07	0.48	0.14

Table 8. Distribution and density % of mosquito across six districts in Mizoram 2009-2011.

The remaining species: *Culex tritaeniorhynchus, Culex mimeticus, Culex bitaeniorhyunchus, Culex tarsalis, Culiseta melanura, Culex peus, Culiseta inornata, Anopheles nivipes*, *Anopheles willmori, Anopheles jeyporiensis, Anopheles minimus, Anopheles dirus* and *Aedes aegypty* were less frequency and regarded as sporadic (C=0-20%). According to the density criterion, three species (*Culex quiquefasciatus, Anopheles barbirostris, Anopheles vagus*) are within the dominant class (D>5%): one of them- *Culex quiquefasciatus* also showed the highest constancy during the study peroid (Table 6). Five species (*Culex mimeticus, Culex tritaeniorhynchus, Culex tritaeniorhynchus, Culex bitaeniorhyunchus* and *Aedes albopictus*) were included in the subdominant class (1< D <5%) while the rest of the twelve species: *Culex tarsalis, Culiseta melanura, Culex peus, Culiseta inornata, Anopheles nivipes*, *Anopheles willmori, Anopheles jeyporiensis, Anopheles minimus,Anopheles dirus* and *Aedes Aegypty, Anopheles jamesi* and *Toxorhynchites splenden* were less dominant and regarded as satellite species (D < 1%) shown in Table 8.

Distribution of mosquito in the surveyed six districts of Mizoram showed that, different species of mosquito were most abundant in Aizawl district with the presence of 14 different species of mosquito (26.92%) followed by 10 species in Mamit districts (18.87%), 9 species in Serchhip district (16.98%). The presence of 8 species each from Lunglei and Kolasib districts comprised 15.09% from the total distribution (figure 12), while the least was found in Lawngtlai district with 4 different species (7.55%).

4.1.5. Seasonal variation of mosquito and abundance in Aizawl

A total of 4948 mosquito individuals (59.41% of total collection) of 14 different species (26.92%) were collected from Aizawl district during 2009-2011 indicated mosquito most abundant and diverse more in Aizawl district (figure 11). Therefore, seasonal-wise mosquito variation and relative abundance was studied in frequently collected mosquito species (Table 9). From the overall collection of mosquito individual (n=4948), it was found that *Anopheles barbirostris* (29.20%) was the dominant species followed by *Culex quinquefasciatus* (27.49%), *Anopheles vagus* (21.40%), *Culex tritaeniorhynchus* (5.11%), *Culex mimeticus* (4.67%), *Aedes albopictus* (4.02%), *Culex bitaeniorhyunchus* (3.27%), *Culex tarsalis* (1.71%), *Culex peus* (1.37%), *Toxorhynchites splenden* (0.57%), *Anopheles philipinensis* (0.56%), *Culiseta melanura* (0.28%) and *Anopheles willmori* (0.12%). Three years survey (2009-2011) data revealed that between the season, the total number of mosquito vary significantly ($P<0.001$), variation in mosquito species was also significant ($P<0.05$). The diversity index (H') and evenness aspect of diversity (H_{eve}) analysis showed that mosquito diversity was highest in Monsoon season (Jul-Sep) and listed in Table 9. The Tukey's test revealed significant differences in abundance of various species of mosquitoes ($P=0.0001$) as given in Table 11. *Anopheles barbirostris* was predominant species and its abundance was significantly different from other mosquito species except *Anopheles vagus* ($q=4.50$; NS) and *Culex quinquefasciatus* ($q=0.62$; NS). The abundance of each mosquito species and their significant differences from other mosquito species was shown in Table 10.

Mosquito species	Sampling period (2009-2011)			
	Jan-mar	Apr-Jun	Jul-Sep	Oct-Dec
Anopheles barbirostris	(0-12) 4.7±1.3	(4-38) 17.2 ±4.3	(6-105) 45.1±18.4	(4-18) 6.6±1.5
Anopheles vagus	(0-34) 11±3.6	(0-24) 9.2±2.46	(0-45) 24.8±2.72	(2-12) 6.2±1.4
Anopheles phillipinensis	-	(0-8) 2.7±1.0	(0-12) 3.3±1.4	-
Culex quinquefasciatus	(2-45) 23.4±5.0	(12-52) 29.7±5.76	(8-85) 46.7±12.1	(4-17) 10.0±1.6
Culex tritaeniorhynchus	-	(0-45) 21.7±4.6	(0-16) 5.7±2.1	(0-14) 7.0±1.8
Culex bitaerhynchus	(0-45) 18.2±5.7	(0-42) 23.8±4.6	(0-62) 25.7±8.4	-
Culex mimeticus	-	(0-45) 15.6±5.4	(0-56) 20.8±8.6	-
Aedes albopictus	(0-32) 9.03±3.6	(0-46) 16±3.6	(0-32) 16.4±4.3	(0-12) 6.6±1.3
Culex peus	-	(0-6) 2.6±0.8	(0-18) 3.7±1.9	-
Culex tarsalis	-	-	(0-21) 7.3±2.6	(0-5) 1.5±0.6
Shannon (H') index	1.843	2.131	2.147	1.991
Evenness index (H_{eve})	0.7016	0.8425	0.8556	0.8133

Table 9. Numbers (Range, mean ± S.E.) of mosquitoes sampled in Aizawl District, Mizoram from 2009 to 2011.

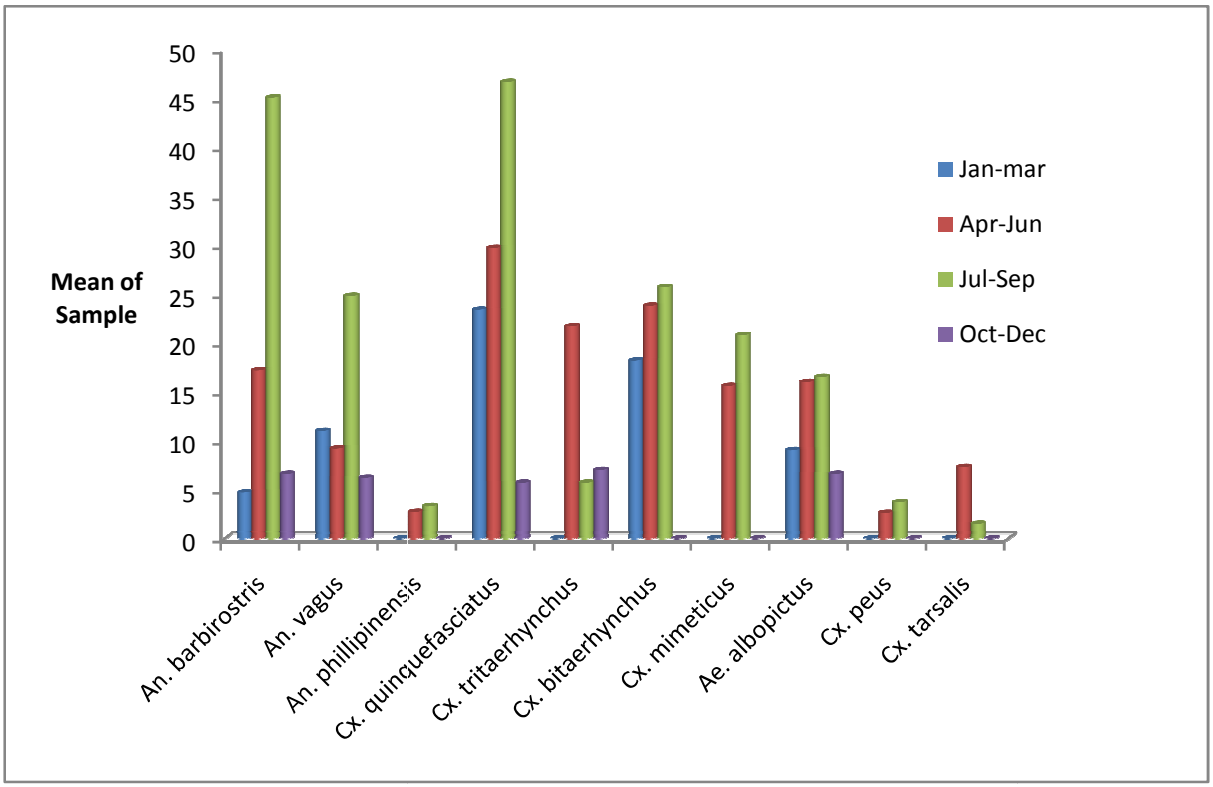


Figure 11. Seasonal variation of mosquitoes sampled in Aizawl district, Mizoram during 2009 to 2011.

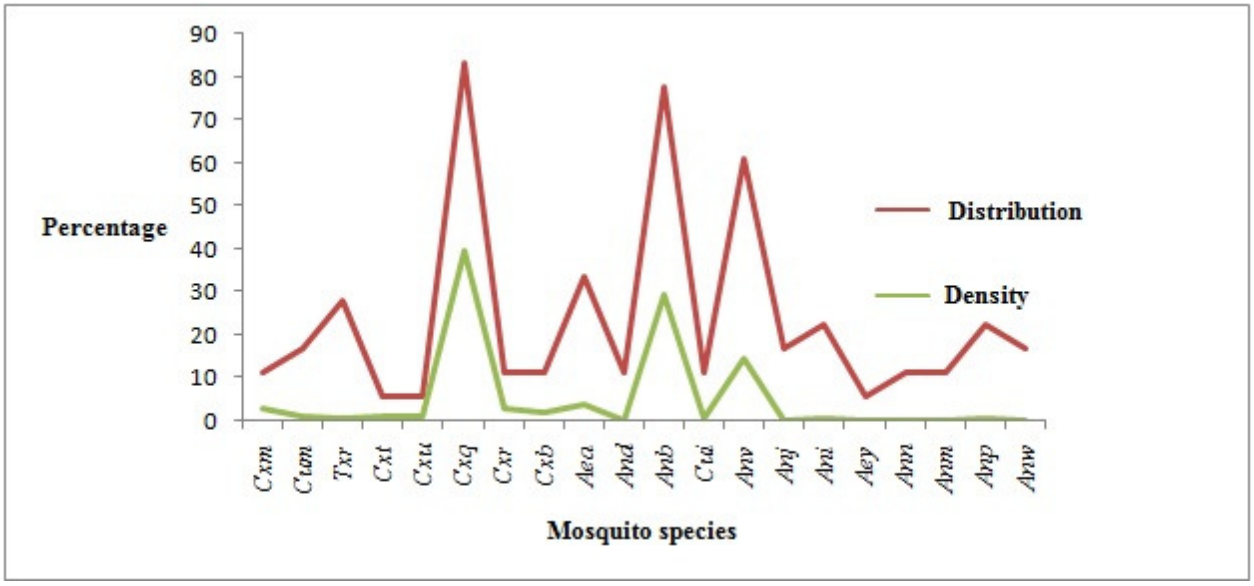


Figure 12. Graph showing distribution and density% of collected mosquitoes across six districts of Mizoram.

Table 10. Results of Tukey test with respect to the abundance of immatures of different mosquito species in Aizawl district.

Tuckey-kramer multiple comparision test:

Comparison	Q=4.52	P-value
Anb vs Anv	4.50	NS
Anb vs Anp	7.83	0.001
Anb vs Cxq	0.62	NS
Anb vs Cxr	5.85	0.01
Anb vs Cxb	5.83	0.01
Anb vs Cxm	7.18	0.001
Anb vs Cxp	8.38	0.001
Anb vs Cxt	8.48	0.001
Anv vs Anp	3.33	NS
Anv vs Cxq	5.13	0.05
Anv vs Cxr	1.35	NS
Anv vs Cxb	1.32	NS
Anv vs Cxm	2.68	NS
Anv vs Cxp	3.88	NS
Anv vs Cxt	3.98	NS
Anp vs Cxq	8.46	0.001
Anp vs Cxr	1.97	NS
Anp vs Cxb	2.00	NS
Anp vs Cxm	0.65	NS
Anp vs Cxp	0.54	NS
Anp vs Cxt	0.65	NS
Cxq vs Cxr	6.48	0.001
Cxq vs Cxb	6.45	0.001
Cxq vs Cxm	7.81	0.001
Cxq vs Cxp	9.01	0.001
Cxq vs Cxt	9.11	0.001
Cxr vs Cxb	0.02	NS
Cxr vs Cxm	1.32	NS
Cxr vs Cxp	2.52	NS
Cxr vs Cxt	2.63	NS
Cxb vs Cxm	1.35	NS
Cxb vs Cxp	2.55	NS
Cxb vs Cxt	2.65	NS
Cxm vs Cxp	1.19	NS
Cxm vs Cxt	1.30	NS
Cxp vs Cxt	0.10	NS

Table 11. Intermediate calculation ANOVA table for mosquito sampled in Aizawl district.

Source of variation	df	SS	MS	F-value	P-value
Treatment (between mosquito species)	8	13878	1734.8	11.76	0.0001
Residuals (within mosquito species)	81	11946	147.48		
Total	89	25825			

Table 12. Mosquitoes sampled (mean value) in Aizawl district.

Rank of sample	Sample mean
Anopheles barbirostris	26.37
Anopheles vagus	12.80
Anopheles phillipinensis	1.50
Culex quinquefasciatus	24.75
Culex tritaeniorhynchus	8.60
Culex bitaerhynchus	16.92
Culex mimeticus	9.10
Aedes albopictus	12.03
Culex peus	1.57
Culex tarsalis	2.4
Standard error	4.33

4.2. Larval breeding habitats and influences of environmental factors on their breeding

Altogether 19 species of mosquitoes collected within the following 5 genera *Aedes, Anopheles, Aedes, Culex* and *Toxorhynchites* were grouped depending on the habitats preferences and shown in Table 13.

While compiling the data with respect of occurrence of immature vs breeding characters, groups I, II, III and IV were distinguished in the phenogram (Figure 13) according to the coefficient of association obtained (Table 13). Group-I includes 8 species: *Culiseta melanura, Culex tarsalis, Culex peus, Aedes albopictus, Culex tritaeniorhynchus, Culex bitaeniorhyunchus, Culex mimeticus and Culiseta inornata,* Group-II includes 6 species: *Anopheles barbirostris, Anopheles vagus, Culex quinquefasciatus, Anopheles jamesii, Anopheles philipinensis* and *Anopheles* nivipes. Group-III includes 3 species: *Anopheles jeyporiensi, Anopheles mimimus* and *Anopheles willmori.* Group-IV includes 2 species: *Aedes aegypty* and *Toxorhynchites splenden.*The highest association coefficient (0.96) was found between *Culex tarsalis* and *Culex peus* (Table 14), followed by *Culiseta melanura* and *Culex tritaeniorhynchus* (0.95). Since, the lowest coefficient of association (0.25) was found between *Culex mimeticus* and *Aedes aegypty* which fall under different group. A unique preference of habitat was found in *Culex tritaeniorhynchus* and *Culex bitaeniorhyunchus* as well as *Culex tarsalis* and *Culex peus* which were on the same group (group I). *Culex quinquefasciatus* had similar breeding preferences as compared to most of the Anopheline species and are placed in groups II while Anopheline species sharing habitats together.

Group	Group-I								Group-II						Group-III			Group-IV	
Variables of breeding habitats	*Cum*	*Cui*	*Cxt*	*Cxu*	*Cxr*	*Cxb*	*Aea*	*Cxm*	*Anv*	*Anb*	*Cxq*	*Anm*	*Anp*	*Ani*	*Anw*	*Ann*	*Anj*	*Aey*	*Txr*
Pond	x	x	x	x	x	x	x	x	x	x	x	-	x	x	-	-	x	-	x
River beds	-	-	-	-	-	-	-	-	x	x	x	-	x	x	x	x	x	-	x
Tanks	-	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	x	x
Stream	-	-	-	-	-	-	-	x	x	x	x	x	-	-	x	-	x	-	-
Ditches	x	-	x	x	x	x	-	x	x	x	x	-	-	-	-	-	-	-	-
Partially shady	-	-	x	x	-	-	x	-	x	x	x	-	-	x	x	x	-	x	x
Sun lighted	x	x	x	x	x	x	x	x	x	x	x	-	x	x	x	x	-	-	-
Quite/stagnant	x	x	x	x	x	x	x	x	x	x	x	-	-	-	-	-	-	x	x
Slow flowing	-	-	-	-	-	-	-	-	x	x	x	x	x	x	x	x	x	-	x
Clear	-	-	-	-	-	-	x	x	x	-	-	x	x	x	-	x	x	x	-
Slightly Turbid	x	-	x	x	x	x	x	x	-	x	x	-	-	-	-	-	-	-	x
Turbid	-	-	x	x	-	-	-	-	-	-	x	-	-	-	-	-	-	-	x
Veg - Moderate	x	x	-	-	x	x	x	x	x	x	x	-	x	x	x	x	-	-	-
Thin	-	-	x	x	-	-	x	-	-	-	x	x	-	-	-	-	-	x	x
Negligible	-	-	-	-	-	-	x	-	x	x	x	-	x	x	-	x	-	x	x
Depth- 0- 1 m	-	-	x	x	-	-	-	-	x	x	x	x	-	x	x	x	x	-	-
1- 2 m	x	x	-	-	x	x	x	-	x	x	x	-	-		-	-	-	x	x
2 m above	x	-	-	-	x	x	x	x	x	x	x	-	x	-	-	-	-	-	-

Table 13. Mosquito larval breeding habitats/characters in respect to different groups of mosquitoes in six different districts of Mizoram (India) during 2009 to 2011.

	Cum	*Txr*	*Cxt*	*Cxu*	*Cxr*	*Cxb*	*Aea*	*Cxm*	*Anb*	*Cxq*	*Cui*	*Anv*	*Anj*	*Ani*	*Aey*	*Ann*	*Anm*	*Anp*	*Anw*
Cum	1																		
Txr	0.45	1																	
Cxt	0.65	0.50	1																
Cxu	0.65	0.60	**0.96**	1															
Cxr	0.95	0.40	0.59	0.60	1														
Cxb	0.94	0.40	0.60	0.60	0.94	1													
Aea	0.70	0.66	0.56	0.55	0.76	0.74	1												
Cxm	0.80	0.24	0.55	0.55	0.84	0.85	0.60	1											
Anb	0.70	0.56	0.55	0.55	0.65	0.65	0.59	0.61	1										
Cxq	0.56	0.70	0.60	0.59	0.50	0.50	0.56	0.46	0.85	1									
Cui	0.85	0.41	0.60	0.60	0.80	0.79	0.65	0.65	0.55	0.40	1								
Anv	0.60	0.46	0.45	0.45	0.54	0.55	0.49	0.60	0.90	0.76	0.55	1							
Anj	0.35	0.30	0.40	0.40	0.31	0.30	0.15	0.45	0.46	0.31	0.50	0.56	1						
Ani	0.50	0.44	0.44	0.44	0.45	0.45	0.50	0.50	0.71	0.55	0.56	0.80	0.65	1					
Aey	0.40	0.66	0.45	0.43	0.35	0.33	0.60	**0.25**	0.32	0.35	0.54	0.39	0.44	0.40	1				
Ann	0.55	0.50	0.59	0.60	0.50	0.50	0.45	0.45	0.65	0.60	0.60	0.54	0.60	0.65	0.35	1			
Anm	0.35	0.40	0.49	0.50	0.30	0.30	0.25	0.46	0.35	0.40	0.50	0.46	0.80	0.56	0.56	0.40	1		
Anp	0.60	0.45	0.34	0.36	0.56	0.55	0.51	0.60	0.60	0.45	0.65	0.70	0.64	0.90	0.41	0.65	0.55	1	
Anw	0.45	0.30	0.50	0.51	0.41	0.40	0.35	0.45	0.65	0.50	0.60	0.65	0.80	0.75	0.35	0.70	0.60	0.65	1

Table 14. Similarity matrix for the operative taxonomic units calculated using coefficient of association.

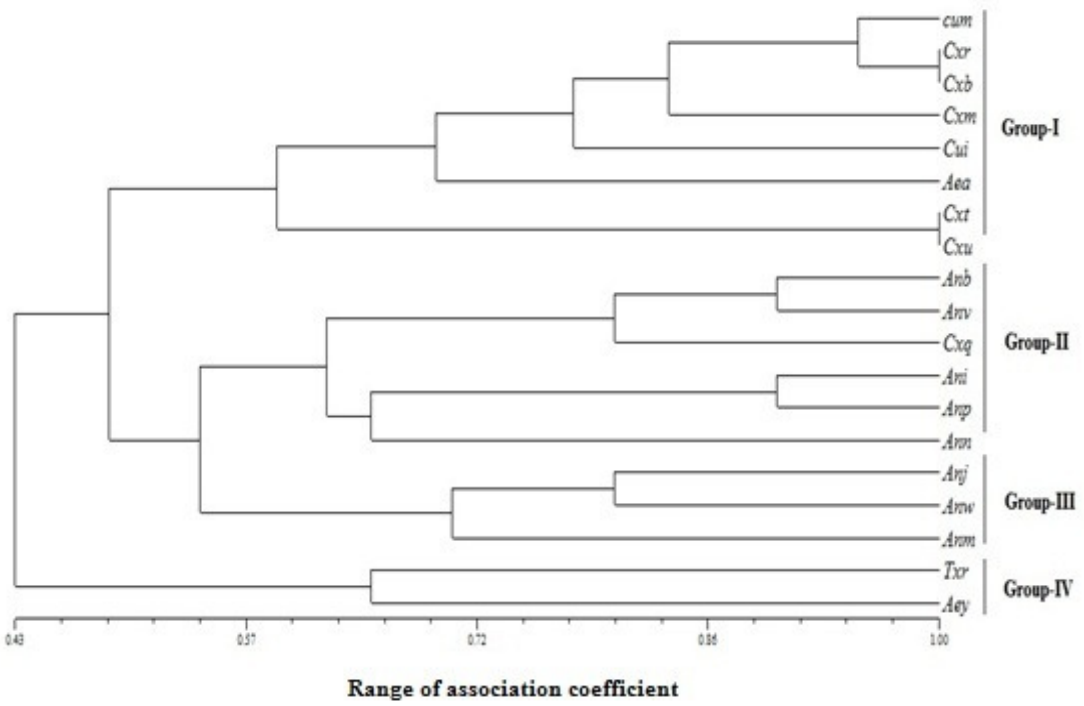


Figure 13. Phenogram of 19 OUT's resulting from Cluster analysis (based on Table 13).

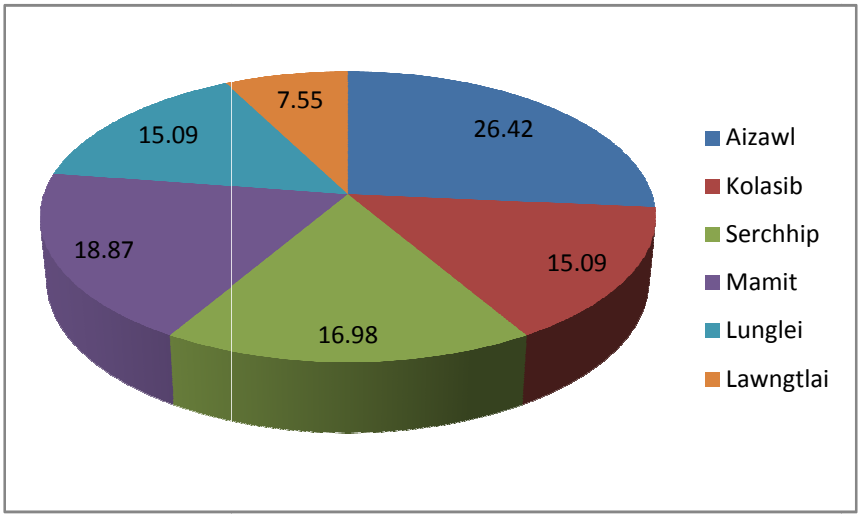


Figure 14. Mosquito abundance (percentile) in six districts of Mizoram.

4.2.1. Characters of breeding habitats

The positive breeding habitats and their quantitative characters (water depth) and qualitative characters (permanent/temporary, shady/lighted, water movement, vegetation condition and turbidity) were listed with regard to presence or absence of different mosquitoes species (Table 10). The breeding habitats of mosquitoes found in the present study were seepage pools, riverbeds, ponds, tanks, ditches, streams, rock holes, tree holes, intradomestic containers and shallow pits. Breeding habitat such as ponds and ditches were the richest habitats sharing 15 mosquito species while the lowest species diversity (3 species) was recorded from tanks. In general, the maximum number of species preferred partially shady and temporary water habitats. Moderate vegetation and slightly turbid water habitats also had higher mosquito species diversity. Mosquito species under Group I were mainly restricted to ponds, seepage pool, rice fields, tanks and ditches. Sun lighted to partially shady and quiet/stagnant habitats with moderate to negligible vegetation at a water depth of 0–2m above were the main characters of the habitats. River beds, streams, ponds, rock holes and pools were the common breeding habitats of the species of Group II. Partially shady habitats both slow flowing or stagnant near river edges and clear to slightly turbid were the main characters of the habitats among group II species. Ponds, river beds and stream were habitats preferences for Group III species. Partially shady to sun lighted with slow flowing and clear habitats with moderate vegetation at the depth of 0-1m were the main breeding characters of these group. Group IV species had unique preferences of tank for breeding. Partially shady with quiet stagnant and clear, negligible vegetation between the depth of 1-2m were the main characters of the habitats. Larvae of *Anopheles barbirostris, Anopheles vagus* and *Culex quinquefasciatus* were found to breed in all of the above stated habitats except in the tank. However, the natural habitats of temporary conditions with shady to partially shady

habitats and moderate vegetation were the main habitats preferences of all collected mosquito species.

4.2.2. Relationship between mosquito larval abundance and environmental variables

The results of forward multiple regression analyzed the effect of environmental parameters on the relative abundance of immature stages of mosquitoes in Aizawl district. The model included 22 variables: 8 physico-chemical variables (Table 15) and 14 species of mosquitoes identified in the samples. Six of the 8 physico-chemical parameters parameters were significantly associated with the relative abundance of immature mosquitoes (Table 16); these included dissolved oxygen, TDS, temperature, alkalinity, pH and hardness.

Anopheles barbirostris was positively associated with pH ($P<0.05$). High alkalinity in the habitat was positively associated with abundance of *Anopheles vagus* ($p<0.05$) while there was a negative association against dissolved oxygen ($p<0.01$). Habitats with alkaline water bodies and slightly turbid with amount of detritus have a positive association against *Culex quinquefasciatus* ($P<0.05$) but *Anopheles philipinensis* larval abundance was negatively associated with temperature ($P<0.05$) and found mostly in small, open, clear water habitats moreover, positively associated with hardness of water and dissolved oxygen ($P<0.05$). There was a positively associated between *Culex tritaeniorhynchus* and alkalinity of water ($P<0.05$). The other environmental variables were excluded in the model because they had weaker associations with mosquito larval abundance (Table 16).

Study site	pH (mg/l)	Alkalinity (mg/l)	Hardness (mg/l)	Temp. (C)	DO (mg/l)	TDS (mg/l)	Phosphate (mg/l)	Chloride (mg/l)
Lengpui	7.3±0.2	93.5±11.5	149.8±3.3	27.1±1.1	5.49±0.07	362.8±20.1	1.3±0.1	6.6±0.4
Dinthar	7.2±0.1	98.0±11.21	176.1±5.2	24.4±1.0	5.42±0.4	199.3±5.7	0.4±0.01	15.6±1.6
Mission-veng	7.0±0.3	48.8±2.7	135.5±12.0	24.7±0.7	7.5±0.5	117.0±19.9	2.5±0.4	3.35±0.5
Ramrikawn	7.1±0.1	52.3±4.5	100±5.0	27±1.0	3.6±0.08	561.0±25.6	2.1±0.2	26.9±1.3
Sihhmui	6.8±0.06	59.4±6.25	101.5±15.9	28.2±0.4	5.5±0.7	126.0±13.5	0.98±0.02	5.7±0.5

Table 15. Average values (mean± SE) of the measured environmental factors (water quality) in Aizawl.

Species	parameter	Co-efficient	P- value
Anopheles barbirostris	pH	0.30	0.018
Anopheles vagus	DO	-0.29	0.038
	Alkalinity	0.07	0.010
Culex quinquefasciatus	TDS	0.002	0.022
	pH	0.28	0.017
	Alkalinity	0.01	0.015
Anopheles philipinensis	Hardness	1.86	0.012
	DO	0.63	0.014
	Temperature	-0.44	0.033
Culex tritaeniorhynchus	Alkalinity	0.08	0.049

Table 16. Linear regression analysis for the abundance mosquito in Aizawl (showing best water quality predictors).

4.3. Lethal bio-assay and susceptibility change in relation to level of metabolic resistant enzyme

Bio-assays were performed on 3rd instar larvae and early adults of *Anopheles barbirostris* and *Anopheles vagus* against different concentrations of insecticides (DDT and Deltamethrin). Based upon susceptibility tests on the field collected population, 1.19 fold increased was seen in the tolerance level to DDT in *Anopheles barbirostris* compared to *Anopheles vagus* in adult tests. Same way, the larvae showed 1.73 fold increased tolerance level to DDT in *Anopheles barbirostris* compared to *Anopheles vagus* that resulted that *Anopheles barbirostris* tolerated more to DDT than *Anopheles vagus* and their LC_{50} were shown in Table 17.

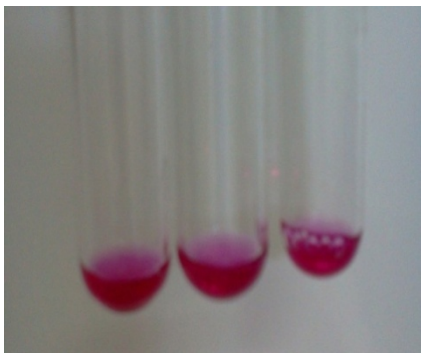
Moreover, in case of deltamethrin susceptibility tests (Table 18), screening of the larvae against insecticides resulted that *Anopheles vagus* (LC_{50}=0.0021) was more susceptible than *Anopheles barbirostris* (LC_{50}=0.0041) while the adults *Anopheles vagus* (LC_{50}=0.0063) was found 1.23 fold increased tolerace level against deltamethrin than *Anopheles barbirostris* (LC_{50}=0.0051).

Larval Species	**Conc (ppm)**	**mortality**	**LC_{50}**	**LCL**	**UCL**	**Adult species**	**Conc (%)**	**mortality**	**LC_{50}**	**LCL**	**UCL**
Anopheles barbirostris	0.004	12	0.387	0.230	0.728	*Anopheles barbirostris*	0.25	8	1.607	1.361	1.937
	0.02	28					0 .50	21			
	0.10	37					1.0	36			
	0.50	54					2.0	52			
	2.5	68					4.0	78			
Anopheles vagus	0.004	18	0.223	0.129	0.418	*Anopheles vagus*	0.25	12	1.340	1.128	1.615
	0.02	32					0.50	28			
	0.10	44					1.0	39			
	0.50	58					2.0	57			
	2.5	78					4.0	81			

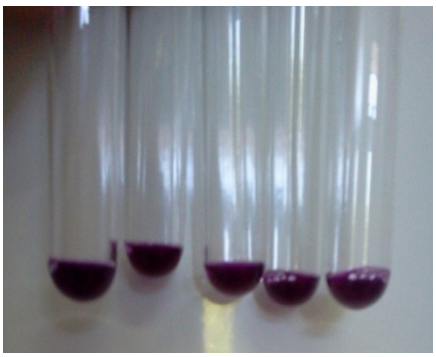
Table 17. Insecticidal bioassays (DDT) against field collected *Anopheles barbirostris* and *Anopheles Vagus*.

Larval Species	Conc (ppm)	mortality	LC_{50}	LCL	UCL	Adult species	Conc (%)	mortality	LC_{50}	LCL	UCL
Anopheles barbirostris	0.002	32	0.0041	0.0041	0.0064	*Anopheles barbirostris*	0.004	48	0.0051	0.0048	0.0052
	0.004	52					0.006	58			
	0.008	66					0.008	69			
	0.01	76					0.010	78			
	0.03	88					0.025	96			
Anopheles vagus	0.002	52	0.0021	0.0012	0.0024	*Anopheles vagus*	0.004	42	0.0063	0.0041	0.0078
	0.004	70					0.006	49			
	0.008	80					0.008	56			
	0.01	86					0.010	63			
	0.03	96					0.025	88			

Table 18. Insecticidal bioassays (deltamethrin) against field collected *Anopheles barbirostris* and *Anopheles Vagus*.

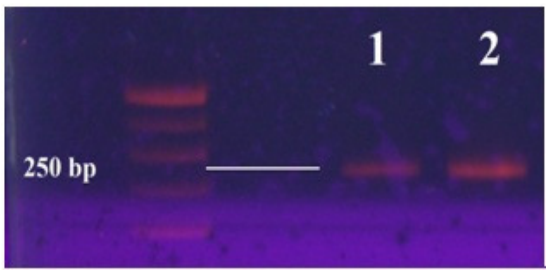


15A. *α*-estearse assays

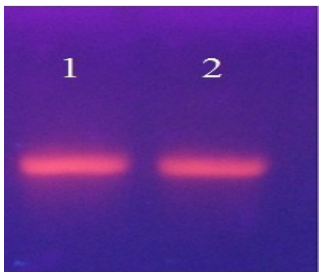


15B. *β*-estearse assays

15C. GST assays



15D.



15E.

Figure 15A-E.positive test tubes for enzyme assays (15A-C). **15D.** 1.5% agarose gel showing standardized β-actin gene qRT-PCR. 100 bp DNA marker was used. 1- *Anopheles vagus*; 2. *Anopheles barbirostris.* **15E.** Gene expression of GSTe4 gene. 1 - *Anopheles vagus*; 2 - *Anopheles barbirostris*

4.3.1 Biochemical assays on insecticides treated samples

The results of the biochemical analysis on insecticides treated samples (alive specimens) showed a similar pattern to bio-assay and there was a significant increase in enzymes production in increasing insecticides concentrations (Table 19). In DDT treated samples, the amount of GST enzyme production was highest in *Anopheles barbirostris* adults (0.420±0.02) and a correlation was found between susceptibility tests on different concentrations of DDT and enzyme elevation ($r=0.953$; $P<0.05$), a similar pattern was found at larval stages in *Anopheles barbirostris* (0.375±0.02) as compared to *Anopheles vagus* (0.196±0.02). Based upon GST enzyme production, it was found that *Anopheles vagus* was significantly more susceptible than *Anopheles barbirostris* against DDT ($P<0.05$). The analysis of general esterase showed heterogeneity in enzyme production. *Anopheles vagus* produced highest esterase (α- 0.110±0.03; β- 0.105±0.02) as compared to others.

In deltamethrin treated samples, GST enzyme production was significantly higher in *Anopheles barbirostris* (0.320±0.02) than *Anopheles vagus* (0.253±0.02). There was a significant correlation of GST enzyme elevation against increasing concentrations of deltamethrin ($P<0.05$). The Spectrophotometric analysis of larval forms revealed that the level of esterases was higher in *Anopheles vagus* (α- 0.035±0.01; β- 0.041±0.01) than *Anopheles barbirostris* (α- 0.021±0.01; β- 0.034±0.01). In contrast, the level of β-esterase was higher in adults of *Anopheles barbirostris* (0.094±0.03) than *Anopheles vagus* (0.072±0.02) but there was no significant correlation between the level of esterases elevation and increasing deltamethrin concentrations in the susceptibility tests (Table 20).

Species	DDT			Deltamethrin		
	General Esterase (α) (α-naphthol/min /mg protein)	General Esterase (β) (β-naphthol/min/ mg protein)	Glutathione - S - transferase (µmoles/min/mg protein)	General Esterase (α) (α-naphthol/min /mg protein)	General Esterase (β) (β-naphthol/min/ mg protein)	Glutathione - S - transferase (µmoles/min/mg protein)
	Mean ±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Anv (L)	0.0801±0.02	0.0735±0.02	0.196±0.02	0.035±0.01	0.041±0.01	0.165±0.01
Anv (A)	0.110±0.03	0.105±0.02	0.375±0.02	0.102±0.03	0.072±0.02	0.253±0.02
Anb (L)	0.096±0.01	0.052±0.01	0.201±0.03	0.021±0.01	0.034±0.01	0.241±0.01
Anb (A)	0.105±0.02	0.084±0.17	0.420±0.02	0.076±0.02	0.094±0.03	0.320±0.02

Table 19. Overall enzyme assays (mean±SE) in insecticides treated samples.

species	insecticides	enzymes	Correlation (r)	r^2	P- value
Anopheles vagus (L)	DDT	GST	0.990	0.981	0.001
Anopheles vagus (A)			0.966	0.934	0.007
Anophelesbarbirostris (L)			0.973	0.947	0.005
Anophelesbarbirostris (A)			0.953	0.908	0.018
Anopheles vagus (L)	Deltamethrin		0.984	0.899	0.012
Anopheles vagus (A)			0.910	0.829	0.035
Anophelesbarbirostris (L)			0.971	0.943	0.005
Anophelesbarbirostris (A)			0.927	0.859	0.021
Anopheles vagus (L)	DDT	α-esterase	0.978	0.957	0.003
Anopheles vagus (A)			0.955	0.912	0.016
Anophelesbarbirostris (L)			0.946	0.931	0.007
Anophelesbarbirostris (A)			0.924	0.852	0.020
Anopheles vagus (L)		β-esterase	0.979	0.958	0.003
Anopheles vagus (A)			0.989	0.968	0.001
Anophelesbarbirostris (L)			0.975	0.950	0.004
Anophelesbarbirostris (A)			0.971	0.943	0.005
Anopheles vagus (L)	Deltamethrin	α-esterase	0.771	0.596	NS
Anopheles vagus (A)			0.653	0.413	NS
Anophelesbarbirostris (L)			0.988	0.911	0.001
Anophelesbarbirostris (A)			0.930	0.866	0.021
Anopheles vagus (L)		β-esterase	0.745	0.632	NS
Anopheles vagus (A)			0.613	0.521	NS
Anophelesbarbirostris (L)			0.961	0.925	0.008
Anophelesbarbirostris (A)			0.721	0.523	NS

Table 20. Correlation between susceptibility tests on different doses of insectides and enzymes (GSTs and esterases) production in treated samples. L-larvae; A-adult.

4.4. Semi-quantitative expression of GSTe4 partial gene

Anopheles vagus and *Anopheles barbirostris* cDNAs were amplified using primers AGSTe4 F and GSTE4 R. Among the different conditions tested, an annealing step of 30s at 58^0C and the use of *Taq* polymerase buffer (1X), $MgCl_2$ (1.5 mM), dNTPs (0.25mM), primer (0.1pM each), *Taq* polymerase (0.5 U), allowed to amplify the GSTe4 region by AGSTe4 F and GSTE4 R primers in two *Anopheles* specimens from the Aizawl district revealed 550 bp fragments on 1.5% (w/v) agarose gel (Figure 15E). A total of 28 PCR was performed for standardization of β-actin partial gene expression. Reverse Transcriptase (RT)-PCR was performed on RNAs from *Anopheles vagus* and *Anopheles barbirostris* using the above mentioned primers. A preliminary monitoring of the PCR revealed that the intensity of actin and GST amplimers increased until 35 amplification cycles, indicating that the PCR reaction is not saturated when the number of cycles is chosen under these values. The expression pattern was established using 35 amplification cycles for actin and cDNA of *Anopheles* GSTe4 respectively. Amplifications performed on non-reverse-transcribed RNA (negative control) did not produce any amplimer, indicating that PCR products were not amplified from any cDNA remaining in the samples.

The result of each semi-quantitative mRNA expression including that of β-actin was repeated three times for its confirmation. The standardized β-actin partial gene qRT-PCR gave the optimum band intensity for field collected *Anopheles* species (Figure 14D) and different volumes of cDNA concentrations: *Anopheles vagus* – 1.2 µl and *Anopheles barbirostris* - 0.8 µl were used for template to obtain similar band intensity. It was obse.rved that *Anopheles vagus*

and *Anopheles barbirostris* were able to express GSTe4 gene (Figure 15E) and therefore confirmed GST enzyme production in the biochemical assay.

4.5. *In-silico* characterization of mosquito GSTs

The results of primary structure analysis suggested that most of the mosquito's GSTe4 proteins were hydrophobic in nature due to the presence of high non-polar residues content (Table 22). The average molecular weight of mosquito's GSTe4 proteins calculated is 572266.9 Da. The computed Iso-electric point (pI) value of EAA07591.6 and EAT42685.1 (pI>7) indicates that these GSTe4 were acidic and the pI of EDS36584.1, AEJ87232.1, AEJ87238.1, ABA02185.1, AEW07374.1 and ACJ64424.1 (pI<7) reveals that these were basic in character. Extinction coefficient of GSTe4 at 280 nm is ranging from 13075 - 259900M^{-1} cm^{-1} with respect to the concentration of Cys, Trp and Tyr. Expasy's ProtParam computes all the values for studied GSTe4 protein sequences because they have Cys, Trp or Tyr residues except in AEJ87238.1. This indicates that these GSTe4 can be analyzed using UV spectral methods (Table 23).

Accession No.	Organism
EAA07591.6	*Anopheles gambiae*
EAT42685.1	*Aedes aegypti*
EDS36584.1	*Culex quinquefasciatus*
AEJ87232.1	*Anopheles stephensi*
AEJ87238.1	*Anopheles funestus*
ABA02185.1	*Anopheles dirus*
ACJ64424.1	*Culex pipiens*

Table 21. GSTe4 protein sequences of mosquitoes retrieved from NCBI database.

Ac. No.	Ala	Arg	Asn	Asp	Cys	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val
EAA07591.6	13.1	4.6	2.9	5.7	1.4	4.0	6.7	6.7	1.7	4.5	7.4	8.9	1.5	2.5	5.7	4.4	6.5	1.7	3.3	6.7
EAT42685.1	10.2	3.8	3.6	5.9	1.4	4.1	7.6	5.3	1.6	5.1	7.3	11	1.6	3.1	5.5	4.4	6.5	1.8	3.1	3.1
EDS36584.1	10.5	3.7	4.4	7.4	1.4	5.3	4.7	5.5	1.5	3.8	10.3	6.3	1.8	6.3	5.9	4.9	4.2	1.4	5.5	5.2
AEJ87232.1	10.7	4.0	3.1	6.2	0.4	2.7	6.2	4.5	2.2	5.8	12.9	8.0	1.3	3.1	5.4	4.9	5.8	0.4	5.8	6.2
AEJ87238.1	9.8	4.5	4.5	6.2	0.9	2.2	6.2	4.5	2.2	7.6	11.2	6.7	1.8	3.6	5.4	7.1	4.5	0	5.4	5.8
ABA02185.1	10	6.8	2.7	5.9	0.9	5.0	7.3	6.8	1.8	3.7	9.6	3.7	2.3	5.0	5.5	2.7	4.1	6.9	6.8	8.2
AEW07374.1	8.9	3.3	3.3	5.1	1.4	4.7	7.0	4.7	2.8	4.2	12.1	5.6	2.3	5.6	6.1	3.7	5.6	0.9	6.1	6.5
ACJ64424.1	8.0	7.1	2.7	3.6	1.8	1.8	9.8	6.2	0.9	8.9	9.8	5.4	1.8	4.5	4.5	8.9	5.4	0.9	4.5	3.6

Table 22. Amino acid composition (in %) of mosquito GSTe4 computed using Expasy's ProtParam tool.

Ac. No.	Organism	Seq.	M. wt	pI	– R	+ R	EC	II	AI	GRAVY
EAA07591.6	*Anopheles gambiae*	1813	198559.5	8.69	225	245	259900	28.92	79.05	-0.407
EAT42685.1	*Aedes aegypti*	1702	190226.8	8.72	230	251	249480	27.91	78.62	-0.504
EDS36584.1	*Culex quinquefasciatus*	730	82411.6	5.37	88	73	115225	32.26	80.67	-0.276
AEJ87232.1	*Anopheles stephensi*	224	25091.6	6.53	28	27	24870	30.48	101.96	-0.137
AEJ87238.1	*Anopheles funestus*	224	25000.7	5.97	28	25	18005	33.40	99.78	-0.125
ABA02185.1	*Anopheles dirus*	223	24975.4	5.30	29	23	33475	31.67	85.53	-0.246
AEW07374.1	*Culex pipiens*	214	24422	5.36	26	19	30495	40.74	91.64	-0.109
ACJ64424.1	*Culex tarsalis*	112	12634.4	5.87	15	14	13075	44.13	91.52	-0.182

Table 23. Parameters of mosquito GSTe4 computed using Expasy's ProtParam tool.

Ac. No.	Structure class			
	Alpha	Beta	Coil	Class
EAA07591.6	64.7	0	35.3	alpha
EAT42685.1	60.9	3.7	35.3	alpha
EDS36584.1	62	0.3	37.7	alpha
AEJ87232.1	71.4	11.7	16.9	mixed
AEJ87238.1	59.1	15.6	25.2	mixed
ABA02185.1	55.7	17.7	26.6	mixed
AEW07374.1	58.3	20.4	21.3	mixed
ACJ64424.1	55.2	16.1	20.7	mixed

Table 24. Percentage of residues forming alpha, beta, and coil structures of GSTe4 computed by SSCP server.

Ac. No.	Percentage of hydrophobic residues	Percentage of hydrophilic residues	Net hydrophobic residues content
EAA07591.6	47.88	45.45	High
EAT42685.1	46.12	48.53	High
EDS36584.1	52.35	42.47	High
AEJ87232.1	52.33	43.3	High
AEJ87238.1	51.57	43.95	High
ABA02185.1	52.97	40.18	High
AEW07374.1	54.21	41.12	High
ACJ64424.1	48.12	45.54	High

Table 25. Hydrophilic and hydrophobic residues content of GSTe4 using Protprop software.

The high extinction coefficient of EAA07591.6, EAT42685.1 and EDS36584.1 indicates presence of high concentration of Cys, Trp and Tyr. The computed protein concentration and extinction coefficients help in the quantitative study of protein- protein and protein-ligand interactions in solution. On the basis of instability index Expasy's ProtParam classifies the AEW07374.1 (40.74) and ACJ64424.1 (44.13) GSTe4 as stable (Instability index > 40) while other GSTe4 as unstable (Instability index < 40). The aliphatic index (AI) which is defined as the relative volume of a protein occupied by aliphatic side chains (A, V, I and L) is regarded as a positive factor for the increase of thermal stability of globular proteins. The very high aliphatic index (AI<70) was found on all GSTe4 infers that these GSTe4 may be stable for a wide range of temperature. Grand Average hydropathy (GRAVY) Index of GSTe4 were ranging from –0·1 to 0·9. The very low GRAVY index was computed on all of GST ranging from -0.1 to -0.5 infers that these GSTe4 could result in a better interaction with water (Table 23).

The secondary structure predicted with the help of program SSCP (Secondary Structural Content Prediction) infers that the EAA07591.6 (64.7%) and EAT42685.1 (60.9%) have rich alanine content and mostly α-helices (Table 24). GSTe4 of EDS36584.1, AEJ87232.1, AEJ87238.1, ABA02185.1, AEW07374.1 and ACJ64424.1 have mixed secondary structure *i.e.* α-helice, β-strands and coils. Protprop software analysis of hydrophobic percentages indicated that GSTe4: EAA07591.6 (47.88%), EAT42685.1 (46.12%), EDS36584.1 (52.35%), AEJ87232.1 (52.33%), AEJ87238.1 (51.57%), ABA02185.1 (52.97%), AEW07374.1 (54.21%) and ACJ64424.1 (48.12%) were hydrophobic and net hydrophobic residues content was high (Table 25). The tool CYS_REC recognized the presence of 24 Cysteines in EAA07591.6, 22 Cysteines in EAT42685.1 and 8 Cysteines in EDS36584.1 sequences and predicted four most

probable SS bond pattern of pairs in EAA07591.6, five probable SS bond pattern of pairs in EAT42685.1 and two probable SS bond pattern of pairs in EDS36584.1 (Table 26). The probable SS bond pattern of pairs were identified using Rasmol were shown in Table 26 and figure 16A-C.

The modelled 3D structures evaluated using the online servers ProQ (Protein Quality predictor server) concluded that according to Maxsub scores: EAA07591.6, EAT42685.1, AEJ87238.1, ABA02185.1, AEW07374.1 and ACJ64424.1 were fairly good model (Maxsub>0.1) while EDS36584.1, AEJ87232.1 and AEW07374.1 were very good model (Maxsub>0.5). Moreover, ProQ structure validation of GSTs based on LG score resulted that: EAA07591.6, EAT42685.1 and ACJ64424.1 were very good model (LG score>2.5) while AEJ87238.1, ABA02185.1, AEW07374.1, EDS36584.1 and AEJ87232.1 were extremely good model (LG score>4) (Table 28).

	CYS_REC	Rasmol
EAA07591.6	103-301 278-292 553-1568 1724-1729	292-362 348-371
EAT42685.1	272-286 273-672 295-1570 943-1613 1458-1618	286-343 342-365 356-387
EDS36584.1	130-635 131-136	12-51

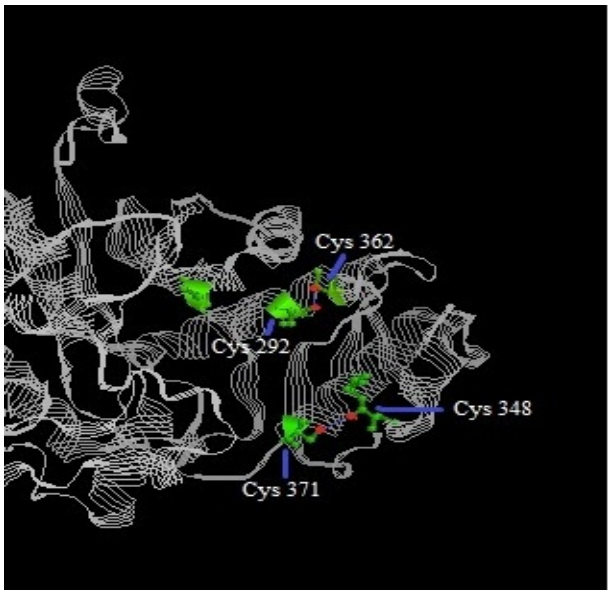
Table 26. Disulphide (SS) bond pattern of pairs predicted by CYS_REC (using primary structure) and identified by Rasmol (using 3D structure modelled). In EDS36584.1, EAA07591.6 and EDS36584.1 GSTe4 sequences.

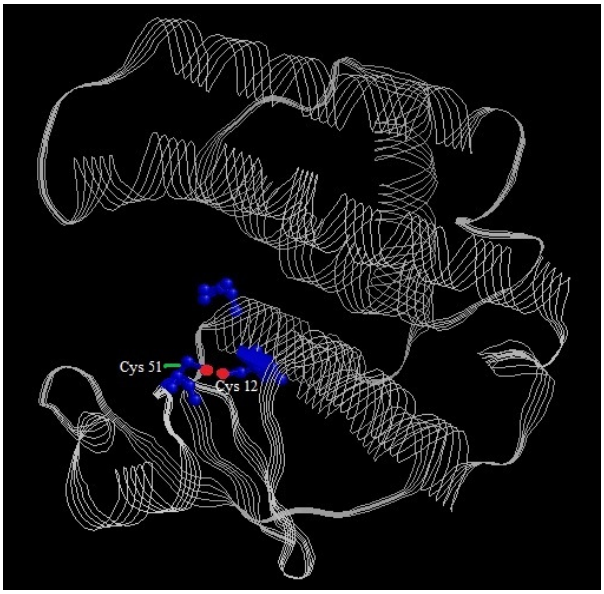
ProQ score		Quality of the model
LG Score	LG Score	
>1·5	>0·1	Fairly good model
>2·5	>0·5	Very good model
>4	>0·8	Extremely good model

Table 27. Criteria for a good (model) 3D structure based on ProQ score.

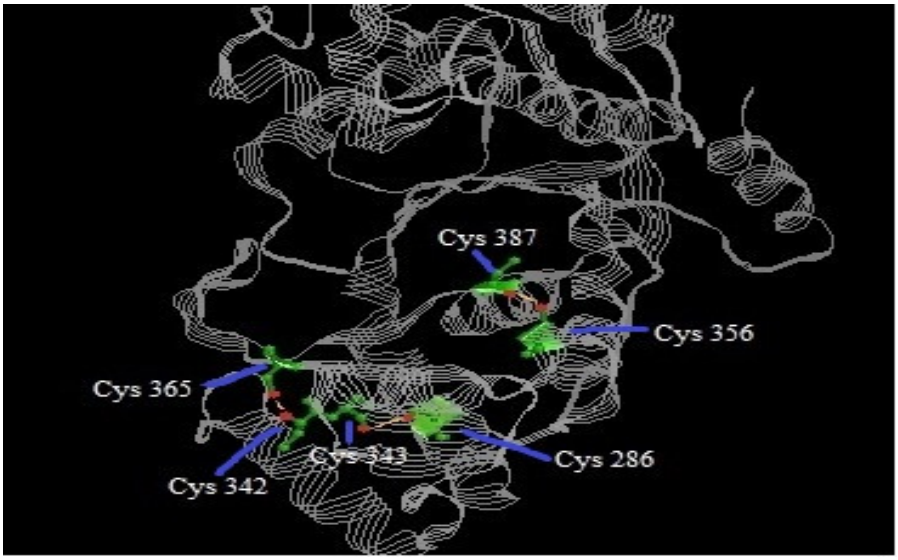
Ac. No.	**ProQ score**	
	LG Score	Maxsub
EAA07591.6	3.651	0.216
EAT42685.1	3.488	0.202
EDS36584.1	5.426	0.502
AEJ87232.1	5.858	0.512
AEJ87238.1	5.215	0.434
ABA02185.1	5.142	0.453
AEW07374.1	5.599	0.582
ACJ64424.1	3.135	0.393

Table 28. Validation parameters computed for the build 3D structures of targets (GSTe4) of mosquitoes using ProQ.





16A. *Anopheles gambiae* **16B**. *Culex quinquefasciatus*



16C. *Aedes aegypti*

Figure 16A-C. RasMol representation (strand) of the homology modelled 3D structures. The sulphur atoms present in cysteines and the disulphide bonds are shown in red colour. Cysteines disulphide bonds positions are marked in blue lines.

V. DISCUSSION

VI. DISCUSSION

5.1. Diversity and relative abundances of mosquito

The present study is the first detailed study on the diversity, distribution and population survey in relation to habitats preference of mosquito in Mizoram. Understanding the abundance and diversity of mosquito species in the area provides an opportunity to better understand the dynamics of vector borne diseases transmission in different ecosystems (Zimmerman, 1992). During the study period, twenty different species of mosquito were collected and identified using taxonomic key prepared for the identification of adult mosquito.

Abundance and distribution of mosquito in the present study showed that *Anopheles* (9 species) was most dominant genus followed by *Culex* (5 species) which was a similar to the survey done by Nagpal and Sharma in1987. Mosquito faunal survey done by Nagpal and Sharma (1987) and Malhotra (1994) reported the prevalence of *Mansonia, Malaya* and *Armigeres* while these mosquitoes were not found during the survey period (2009-2011) but the present study reported the prevalence of *Culiseta* (*Culiseta melanura* and *Culiseta inornata*) which was the first report so far.

A detailed report on the species abundance and distribution showed that *Anopheles dirus* (0.03%) and *Anopheles minimus* (0.07%), a primary vector of malaria (Das and Baruah, 1985) were found in less numbers while *Anopheles barbirostris* (29.50%) a predominant species among *Anopheles,* a probable malaria vector (Limrat *et al*., 2001) and *Anopheles philipinensis,* a secondary vector of malaria (0.56%) were found in malaria endemic areas. Therefore, there may be a chance of vector transmission. *Culex quinquefasciatus* (vector of filariasis) was found throughout the year, regarded as constant (C=80.1-100%) and dominant class (D>5%) suggested

that there may be a chance of filarial incidences and transmission in Mizoram as the neighboring states were already infected by filarial diseases (NVBDCP, 2013). A similar pattern was found the prevalence of *Culex tritaeniorhyncus* (3.03%) vector of Japanese encephalistis but no report of positive case till date (Health Dept. Govt. of Mizoram, 2012). In 2012, there were 12 positive cases of dengue fever (infected from outside) (Health Dept. Govt. of Mizoram, 2012). Therefore, the Health Dept. Govt. of Mizoram has started new initiative of dengue dectecting centre in sentinental hospital (Aizawl) and training of detection and diagnosis of dengue was conducted in all districts on Mizoram thereby neglecting vector control. The present study also found the presences of dengue vectors *Aedes aegypti* (0.02%) and *Aedes albopictus* (3.63%) in Mizoram. Therefore, more vector control measure was needed to initiate to prevent from vector borne diseases transmission.

5.2. Habitats characteristics of mosquito

Both quantitative and qualitative characters of the mosquito breeding habitats have contributed to understanding the similarity of habitat requirements of different species (Devi and Jauhari, 2007). Almiron and Brewer (1996) pointed out that, different types of habitats, both natural and artificial, nature of vegetation, water movement and water depth were the main characters that explain the observed variations among mosquito species. Cluster analysis done by Almiron and Brewer (1996) based on habitat similarity reported that four groups of species have been associated which is similar to the present study that 4 groups are recorded. The phenogram proposed by Almiron and Brewer (1996) with 19 operative taxonomic units, is similar with that found in the present study that has 19 operative taxonomic units but different from the phenogram proposed by Devi and Jauhari (2007) which has 23 operative taxonomic units.

There are a number of papers on the relationship between vegetation and immature stages by several authors (Savage *et al*., 1990; Rajmankova *et al.*, 1992; Rodriguez *et al*., 1993; Rajnikant *et al*., 1996) and almost all of them reported that larval abundance is related to the presence of a particular kind of vegetation. Their results get support from Adityaa *et al*. (2006) who found cemented temporary pools containing maximum food resources, in term of detritus, vegetation and algae allowing the maximum number of species of different guilds to coexist but in the present study, most of the immature mosquitoes were collected from ponds, ditches and river beds. In the present study, immature mosquito found in turbid water were almost always Culicines, which is similar to the findings of Sattler *et al.* (2005). The preference of Anophelines immatures to breed in clear to slightly turbid water is similar to the findings of Bates (1947) and Robert *et al*. (1998). However, Gimning *et al*. (2001) found *Anopheles gambiae* larval densities with turbid water bodies. Further, the results of the present findings are contrast to those of Minakawa *et al*. (1999) and Edillo *et al*. (2002) in having different mosquito species as well as fluctuating ecological conditions prevailing in the area.

Considering the results of the present study in comparison to earlier findings, it has been found that positive associations between mosquito species may result from a common preference for a particular habitat. Maximum immature associations, as recorded in the habitats such as ponds, ditches and river beds, suggest high survival rate, ovipositional preferences and favorable physicochemical characteristics of these habitats. It was also noticed that prolonged water logging with fast changing ecological conditions and extensive surface area of habitats offered favorable breeding conditions to a number of mosquito species including disease vectors. The co-existence of more than one species in a habitat at a given time indicated that mosquito species of the same nature and preference interact with each other (Devi and Jauhari, 2007). Thus,

Understanding mosquito habitats ecology is, therefore, important in designing vector borne diseases control programmes (Adityaa *et al*., 2006).

Seasonal variation of mosquito species found in the study was due monsoon that influences temperature, humidity and temporary breeding habitats, which is essential for mosquito survival (Devi and Jauhari, 2008). Apart from these, rainfall not only provides the medium for the aquatic stages of the mosquito's life but also increases the relative humidity and thereby increasing the longevity of the adult mosquitoes (Michael and Martens, 1995). Therefore, the abundance was increasing during pre-monsoon (Apr-Jun) and the peak was found during monsoon season (Jul-Sep) and gradually declined at post-monsoon.

5.3. Effect of environmental factors on mosquito breeding

Mosquitoes use chemical and biological cues to detect the water quality for ovipositing in habitats (Blaustein and Kotler, 1993). Several studies have examined the influence of dissolved oxygen concentration on the abundance of *Anopheles* spp. and *Culex* spp. with contradicting results (Muturi *et al*., 2006). Grillet (2000) reported a positive association between dissolved oxygen and the abundance of *Anopheles oswaldoi.* Sunish *et al*. (2006) suggested that high algal productivity and associated photosynthesis is responsible for high dissolved oxygen concentrations in aquatic habitats, thereby favoring higher survival of mosquito larvae. In the current study, dissolved oxygen was a best significant factor in productivity *of Anopheles philipinensis and Anopheles vagus* in habitats. Total dissolved solids (TDS), which is the sum of all dissolved organic, inorganic, and suspended solids in water was also a significant factor in larval abundance of *Culex quinquefasciatus* ($p<0.012$). In most areas of its distribution, *Culex quinquefasciatus* prefer habitats rich in dissolved matter and such habitats tend to have high TDS (Hassan *et al*., 1993). *Culex tritaerhynchus and Culex quinquefasciatus* which were mainly

collected from ponds containing alkaline water bodies, which shows they can tolerated alkaline habitats. The breeding of *Anopheles* were small, open and clear habitats characterized by high dissolved oxygen, low nutrient levels and normally good quality water (Muturi *et al*., 2006) and this confirmed the assertion that, *Anopheles philipinensis* had a significant productivity of hardness ($P<0.05$) and dissolved oxygen ($P<0.05$). Besides in small and open habitats, larval predation was less prevalent in temporary habitats than in large permanent habitats (Shililu *et al*., 2003) and finally, open habitats that tend to produce more algae (the main food source for Anopheline spp).

5.4. Insecticides susceptibility status

Insecticide resistance can be due to selection of changes in insect enzyme systems, leading to rapid detoxification or sequestration of insecticide or due to alterations of the insecticide target site preventing the insecticide-target site interaction. Increased metabolic capacity is usually achieved by increased activity of monooxygenases, GSTs or esterases. GSTs can mediate resistance to organophosphates, organochlorines and pyrethroids. Esterases can provide resistance to organophosphates, carbamates and pyrethroids which are rich with ester-bonds (Feyereisen, 2005; Li *et al*., 2007). The insecticidal bioassay and biochemical assays in the present study found the level of tolerant against DDT was higher in *Anopheles barbirostris* than *Anopheles vagus* but both species were susceptible to deltamethrin even there was no significant correlation of the biochemical assays. Therefore, it was found that based upon susceptibility tests there was a possibility of resistant on DDT (WHO, 1998) in *Anopheles barbirostris* and *Anopheles vagus* that needs to confirm. IRS (Insecticide Residual Spray) of DDT was initiated in Mizoram since 1960s (Health dept. Govt. of Mizoram, 2012). Thus, the continuous and indiscriminate use of insecticide in a population will lead to the development of physiological

resistance in the insects (Ganesh *et al*., 2003). This fact could be the reason for both Anopheline species being slightly tolerant to DDT as it was observed in the insecticidal bioassay and biochemical results that there was a significant elevation of GSTs and general esterase (α- and β-esterase) enzyme activity in increasing insecticides (DDT) concentrations. Thus, high resistance levels of DDT in all populations probably are due to increased levels of GST enzymes. (Perera *et al*., 2008). Therefore, in mosquitoes, the metabolic resistance based on GST is the major mechanism of DDT-resistance (Hemingway and Ranson, 2000).

Moreover, GST-based resistance has been detected by elevated levels of GST activity in strains of insects resistant to organophosphates (Fournier *et al.*, 1992), organochlorines (Grant and Hammock, 1992) and pyrethroids (Kostaropoulos *et al.,* 2001). In addition to these, there was a significant elevation of GST enzymes production in increasing deltamethrin concentrations in treated species but insignificant correlation of elevation of general esterase (*α*- and *β*-esterase). Thus, GST alone detoxification was responsible for slightly tolerant against deltamethrin but elevated pyrethroid tolerance in *Anopheles* was because of increased level of esterase (Ganesh *et al*., 2003). Therefore, *Anopheles barbirostris* and *Anopheles vagus* were characterized as susceptible against pyrethroid (deltamethrin).

5.5. Expression of GSTe4 gene

The biochemical assays were only a simple method for detecting the quantity of the resistant enzyme in mosquito population. It is needed of molecular confirmation whether the resistant gene (GST) expressed or not. Thus, the study of mRNA expression profile and *in-vitro* expression of GST gene in *Anopheles* confirmed insecticidal bioassays and biochemical assays

of GST. Therefore, *Anopheles barbirostris* and *Anopheles vagus* were characterized as tolerated against DDT and there was possibility of resistance that needs to confirm (WHO, 1998).

5.6. *In-silico* characterization of mosquito GSTe4

Computational tools and online server characterized mosquito GSTe4 as hydrophobic in nature that may be due to presences of high non-polar residues. Computed GSTe4 protein were mostly basic in nature, can be analyzed using UV spectral methods. They were stable for a wide range of temperature and having a properties of better interaction with water. The SS bonds predicted from the primary structure (protein sequence) using CYS_REC tool migt not be correct and the SS bonds identified from the three-dimensional structure (3D coordinates) using the Rasmol tool might be correct. According to ProQ validation, the modelled 3D structures were characterized as fairly good model (Maxsub score) and extremely good model (LG score).

VII. SUMMARY

VI. SUMMARY

- ✓ The present study detailed on survey of mosquito of Mizoram and their relationship with its occurrence and their breeding habitats. Taxonomic key was prepared for the identification of adults mosquito. A total of 8328 mosquitoes collection representing the 5 genera of: *Anopheles*, *Aedes*, *Culiseta*, *Culex* and *Toxorhynchites* altogether of 20 species were collected. The most dominant genus was *Anopheles* followed by *Culex*, *Culiseta*, *Aedes* and *Toxorhynchites.*

- ✓ Distribution and diversity studies showed the following pattern as *Culex quinquefasciatus* (39.67%), *Anopheles barbirostris* (29.50%), *Anopheles vagus* (14.51%), *Aedes albopictus* (3.62%), *Culex tritaeniorhynchus* (3.03%), *Culex mimeticus* (2.78%), *Culex bitaeniorhyunchus* (1.94%), *Culex tarsalis* (1.02%), *Culiseta melanura* (0.80%), *Culex peus* (0.82%), *Toxorhynchites splenden* (0.64%), *Anopheles philipinensis* (0.48%), *Culiseta inornata* (0.33%), *Anopheles jamesi* (0.29%), *Anopheles nivipes* (0.19%), *Anopheles willmori* (0.14%), *Anopheles jeyporiensis* (0.10%), *Anopheles minimus* (0.07%), *Anopheles dirus* (0.03%) and *Aedes aegypty* (0.02%). *Culex quinquefasciatus* was a dominant species and found throughout year followed by *Anopheles barbirostris*. The present study found the prevalence of *Culiseta melanura* and *Culiseta inornata* which were the first report in Mizoram.

- ✓ Relative abundance studies in different districts of Mizoram suggested that: *Culex quiquefasciatus* was mostly found throughout year and regarded as constant (C=80.1-100%). Two species (*Anopheles barbirostris* and *Anopheles vagus*) were frequent in most of the months (60.1 -80%) while four species: *Aedes albopictus, Anopheles philipinensis,*

Anopheles jamesi, Toxorhynchites splenden were considered as infrequent (20.1-40%). The remaining species: *Culex tritaeniorhynchus, Culex mimeticus, Culex bitaeniorhyunchus, Culex tarsalis, Culiseta melanura, Culex peus, Culiseta inornata, Anopheles nivipes*, *Anopheles willmori, Anopheles jeyporiensis, Anopheles minimus, Anopheles dirus* and *Aedes aegypty* were less frequency and regarded as sporadic (C=0-20%). According to the density criterion, three species (*Culex quiquefasciatus, Anopheles barbirostris, Anopheles vagus*) are within the dominant class (D>5%); one of them, *Culex quiquefasciatus,* also showed the highest constancy during the study peroid. Five species (*Culex mimeticus, Culex tritaeniorhynchus, Culex tritaeniorhynchus, Culex bitaeniorhyunchus* and *Aedes albopictus*) were included in the subdominant class (1< D <5%) while twelve species: *Culex tarsalis, Culiseta melanura, Culex peus, Culiseta inornata, Anopheles nivipes*, *Anopheles willmori, Anopheles jeyporiensis, Anopheles minimus,Anopheles dirus* and *Aedes Aegypty, Anopheles jamesi* and *Toxorhynchites splenden,* were less dominant and regarded as satellite species (D < 1%).

- ✓ *Anopheles dirus* (0.03%) and *Anopheles minimus* (0.07%), a primary vector of Malaria (Das and Baruah, 1985), were found in less numbers while *Anopheles barbirostris* (29.50%), a incriminated malaria vector was predominant species and even found in malaria endemic study area. Therefore, there may be a chance of vector transmission.

- ✓ The survey found the presence of mosquito vector complexes in Mizoram, as there is a chance of other vector borne diseases transmission in Mizoram (the neighboring states

were already infected by these diseases) as dengue infection started in Mizoram since 2012.

- ✓ Seasonal-wise mosquitoes variation and relative abundance studies in Aizawl district shows that from the overall collection of mosquito individual (n=4948), it was found that *Anopheles barbirostris* (29.20%) was the dominant species, followed by *Culex quinquefasciatus* (27.49%), *Anopheles vagus* (21.40%), *Culex tritaeniorhynchus* (5.11%), *Culex mimeticus* (4.67%), *Aedes albopictus* (4.02%), *Culex bitaeniorhyunchus* (3.27%), *Culex tarsalis* (1.71%), *Culex peus* (1.37%), *Toxorhynchites splenden.* (0.57%), *Anopheles philipinensis* (0.56%), *Culiseta melanura* (0.28%) and *Anopheles willmori* (0.12%). Three years survey (2009-2011) data revealed that between the season, the total number of mosquito vary significantly ($P<0.001$), variation in mosquito species was also significant ($P<0.05$). The diversity index (H') and evenness aspect of diversity (H_{eve}) analysis showed that mosquito diversity was highest in Monsoon season (Jul-Sep).The Tukey's test revealed significant differences in abundance of various species of mosquitoes ($P = 0.0001$). *Anopheles barbirostris* was predominant species and its abundance was significantly different from other mosquito species.

- ✓ Based upon habitats preferences, collected mosquitoes were distinguished into four groups according to the coefficient of association obtained. Group-I includes 8 species: *Culiseta melanura, Culex tarsalis, Culex peus, Aedes albopictus, Culex tritaeniorhynchus, Culex bitaeniorhyunchus, Culex mimeticus and Culiseta inornata,* Group-II includes 6 species: *and Anopheles barbirostris, Anopheles vagus, Culex*

quinquefasciatus, Anopheles jamesii, Anopheles philipinensis and *Anopheles* nivipes. Group-III includes 3 species: *Anopheles jeyporiensi, Anophelesmimimus* and *Anopheles willmori.* Group-IV includes 2 species: *Aedes aegypty* and *Toxorhynchites splenden.*The highest association coefficient (0.96) was found between *Culex tarsalis* and *Culex peus*, followed by *Culiseta melanura* and *Culex tritaeniorhynchus* (0.95). Since, the lowest coefficient of association (0.25) was found between *Culex mimeticus* and *Aedes aegypty* which fall under different group. The breeding habitats of mosquito species found in the present study were seepage pools, riverbeds, ponds, tanks, ditches, streams, rock holes, tree holes, intradomestic containers and shallow pits. Breeding habitat such as ponds and ditches were the richest habitats sharing 15 different mosquito species while the lowest breeding habitats of mosquito species (3 species) was recorded from tanks. Natural habitats of temporary conditions with shady to partially shady and moderate vegetation were the main habitats preferences of collected mosquito species.

- ✓ Studies on relationship between mosquito larval abundance and environmental variables resulted that: pH, Dissolved Oxygen, Alkalinity, Temperature, Total Dissolved Solid and Hardness of water were the main biotic factors that influenced the breeding of mosquito species. *Anopheles barbirostris* was positively associated with pH ($P<0.05$). High alkalinity in the habitat was positively associated with abundance of *Anopheles vagus* ($p<0.05$) while there was a negative association against dissolved oxygen ($p<0.01$). Habitats with alkaline water bodies and slightly turbid with amount of detritus has a positive association against *Culex quinquefasciatus* ($P<0.05$) but *Anopheles philipinensis* larval abundance was negatively associated with temperature ($P<0.05$) and found mostly

in small, open, clear water habitats thus, positively associated with hardness of water and dissolved oxygen ($P<0.05$). There was a positively associated between *Culex tritaeniorhynchus* and alkalinity of water ($P<0.05$).

- ✓ Based upon susceptibility tests on the field collected population, *Anopheles vagus* (LC_{50}=1.340) was more susceptible against DDT than *Anopheles barbirostris* ((LC_{50}=1.607) while in case of deltamethrin susceptibility tests, *Anopheles vagus* (LC_{50}=0.0063) was found 1.23 fold increased tolerace level against deltamethrin as compared to *Anopheles barbirostris* (LC_{50}=0.0051). Thus, *Anopheles barbirostris* was able to tolerate more against DDT than *Anopheles vagus* while the level of tolerance against deltamethrin was higher in *Anopheles vagus.* Therefore, it was found that based upon susceptibility tests there was a possibility of resistant on DDT (WHO, 1998) in *Anopheles barbirostris* and *Anopheles vagus* that needs to confirm.

- ✓ Biochemical assays resulted that, In DDT treated samples, the amount of GST enzyme production was highest in *Anopheles barbirostris* (0.420±0.02) and there was a correlation between increasing concentrations of DDT and enzyme elevation ($r = 0.953$; $P<0.05$). A similar pattern was also found in esterase assays that, esterase elevation was found correlated with increasing concentrations of DDT. In deltamethrin treated samples, GST enzyme production was higher in *Anopheles barbirostris* (0.320±0.02) than *Anopheles vagus* (0.253±0.02). There was a significant correlation of GST enzyme elevation and increasing concentrations of deltamethrin ($P<0.05$) while the level of esterases production was higher in *Anopheles vagus* (α- 0.035±0.01; β- 0.041±0.01) than *Anopheles*

barbirostris (α- 0.021±0.01; β- 0.034±0.01) but there was no significant correlation between the level of esterases elevation and increasing deltamethrin concentrations.

- ✓ A total of 28 PCR was performed for standardization of β-actin partial gene expression. The result of each semi-quantitative mRNA expression including that of β-actin was repeated three times for its confirmation. The standardized β-actin partial gene qRT-PCR gave the optimum band intensity for field collected *Anopheles* species. From the expression study of mosquito GSTe4, it was observed that *Anopheles vagus* and *Anopheles barbirostris* were able to express GSTe4 gene.

- ✓ Computational tools and online server characterized mosquito GSTe4 as hydrophobic in nature that may be due to presences of high non-polar residues. Computed GST e4 protein were mostly basic in nature, can be analyzed using UV spectral methods. They were stable for a wide range of temperature and having a properties of better interaction with water. The SS bonds predicted from the primary structure (protein sequence) using CYS_REC tool might not be correct and the SS bonds identified from the three-dimensional structure (3D coordinates) using the Rasmol tool might be correct. According to ProQ validation, the modelled 3D structures were characterized as fairly good model based on Maxsub score and extremely good model based on LG score.

VII. RECOMMENDATIONS

VII. RECOMMENDATIONS

- Further research was needed to confirm the role of incriminate malaria vectors in Mizoram, as there may be vector status changes.

- An indepth study is needed regarding the bioecology, breeding habitats, and vector role transmission.

- There is a chance of other vector borne diseases occurrences in Mizoram not only malaria. Therefore, more vector management programme (biological control: *Bacillus thuringiensis israeliensis, Bacillus sphaericus* and other classes of insecticides: temephos, malathion etc., which was already implemented in neighboring states) is needed to initiated to prevent from diseases transmission.

- Further research was needed to confirm DDT resistance in *Anopheles barbirostris* (a incriminated malarial vector).

VIII. BIBLIOGRAPHY

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A

Abbott, W.S. (1925) A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18, 265–266.

Almiron, W.R. and Brewer, M.E. (1994) Immature stages of mosquitoes (Diptera, Culicidae) collected during the autumn-winter period in Cordoba Province, Argentina. *Mem. Inst. Oswaldo Cruz.* 89, 625-628.

Almiron, W.R. and Brewer, M.E. (1996) Classification of immature stage habitats of Culicidae (Diptera) collected in Córdoba, Argentina. *Mem. Inst. Oswaldo Cruz.* 91, 1-9.

Amerasinghe, F., Indrajith, N. and Ariyasena, T. (1995) Physico-chemical characteristics of mosquito breeding habitats in an irrigation development area in Sri Lanka. Ceylon *J. Sci.* 24, 13-29.

American Public Health Association (1992). *Standard Methods for the Examination of Wastes and Wastewater*, Washington, DC: American Public Health Association.

Adityaa, G., Pramanika, M.K. and Sahaa, G.K. (2006) Larval habitats and species composition of mosquitoes in Darjeeling Himalayas, India. *J. Vect. Borne Dis*. 43, 7–15.

Armstrong, R. N. (1997) Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chem. Res. Toxicol*. 10, 2–18.

Almiron, W.R. and Brewer, M.E. (1996) Classification of immature stage habitats of Culicidae (Diptera) collected in Cordoba, Argentina. *Mem. Inst. Oswaldo Cruz.* 91, 1-9.

Armstrong, R.N. (1991) Glutathione S-transferases: reaction mechanism, structure, and function. *Chem. Res. Toxicol.* 4, 131–140.

Adler, V., Yin, Z., Fuchs, S.Y., Benezra, M., Rosario, L.and Tew, K.D. (1999) Regulation of JNK signaling by GSTp. *Embo. J.* 18, 1321-1334

Apperson, C.S. and Georghiou, G.P. (1975) Mechanisms of resistance to organophosphorus insecticides in Culex tarsalis. *J. Eco. Entomol*. 68(2), 63–78.

B

Brogdon, W. G., McAllister, J. C., Corwin, A. M. and Cordon-Rosales, C. (1999) Oxidase-based DDT-pyrethroid cross-resistance in Guatemalan *Anopheles albimanus*. *Pes. Biochem. Physiol.* 64, 265-272.

Bates, M. (1947) The development and longevity of haemagogus mosquitoes under laboratory condition. *Entomol. Soc. America.* 1, 1-12.

Beall, C., Fyrberg, C., Song, S. and Fyrberg, E. (1992) Isolation of a *Drosophila* gene encoding glutathione S-transferase. *Biochem. Genet*. 30, 515- 527.

Board, P. G., Coggan, M., Chelvanayagam, G., Easteal, S., Jermiin, L. S., Schulte, G. K., Danley, D. E., Hoth, L. R., Griffor, M. C. and Kamath, A. V. (2000) Identification, characterization, and crystal structure of the Omega class glutathione transferases. *J. Biol. Chem*. 275, 24798–24806.

Bloomquist, J.R. (1998) Chemistry and toxicology of the chlorinated cyclodienes and lindane. *Rev. Pestic. Toxicol.* 2, 333-355.

Blaustein, L. and B. Kotler. (1993) Oviposition habitat selection by the mosquito, *Culiseta longiareolata*: Effects of conspecifics, food and green toad tadpoles. *Ecol. Entomol*. 18, 104-108.

Bhargava, M. M., Listowsky, I., Arias, I. M. and Ligandin. (1978) Bilirubin binding and glutathione S-transferase activity are independent processes. *J. Biol. Chem*. 253,4112–4115.

Barraud, P.J. (1934) *The Fauna of British India including Ceylon and Burma*. v. V. London, UK: Taylor and Francis. p. 1–463.

Basio, R.G., White, D.W. and Reisen, W.K. (1970) On Philippine mosquitoes II. Observations on the ecology of mosquitoes of Mt. Makiling and its environs in Luzon, Philipp. *Entomol.* 1, 431–451.

Bayoh, M.N. and Lindsay, S.W. (2003) Effect of temperature on the development of the aquatic stages of *Anopheles gambiae* sensu stricto (Diptera: Culicidae). *Bul. Entomol. Res*. 93, 375-81.

Bhat, H.R. (1975) A survey of haematophagous arthropods in western Himalayas, Sikkim and hill districts of West Bengal : records of mosquitoes collected from Himalayan Region of Uttar Pradesh with ecological notes. *Indian J. Med. Res.* 63(11), 1583–608.

Bhatt, R.M., Sharma, R.C., Srivastava, H.C., Gautam, A.S. and Gupta, D.K. (1993) Interspecific associations among Anophelines in different breeding habitats of Kheda district Gujarat: Part II-Non-Canal area. *Indian J. Malariol.* 30, 91-100.

Bogwitz, M. (2005) The genetics resistance to lufenuron in *Drosophila melanogaster*. Ph.D. Thesis, University of Melbourne, Melbourne, Australia .

Brown, A.W.A. (1986) Insecticide resistance in mosquitoes: a pragmatic review. *J. Am. Mosq. Control Assoc*. 2, 123-140.

Boddington, R.G. (1992) Characterization of malathion carboxylesterases and nonspecific esterases in the malaria mosquito *Anopheles stephensi*. PhD. diss. Univ. London

C

Courtney, E.G. and Robert, F. G. (2004) Proteomics computational analyses suggest that the bornavirus glycoprotein is a class III viral fusion protein (γ penetrene). *Virol. J.* 6, 145-155.

Christophers, S.R. (1933) *The Fauna of British India including Ceylon and Burma*. v. IV. London, UK: Taylor and Francis 1933. p. 1–360.

Chen, X., Yang, X., Senthil Kumar, N., Tang, B., Sun, X., Qiu, X. and Zhang, W. (2007) The class A chitin synthase gene of *Spodoptera exigua*: Molecular cloning and expression patterns. *Insect Biochem. Mol. Biol.* 37, 409–417.

Cho, S.G., Lee, Y.H., Park, H.S., Ryoo, K., Kang, K.W., Park, J., Eom, S.J., Kim, M.J., Chang, T.S., Choi, S.Y., Shim, J., Kim, Y., Dong, M.S., Lee, M.J., Kim, S.G., Ichijo H and Choi, E.J. (2001) Glutathione S-transferase Mu modulates the stress-activated signals by suppressing apoptosis signalregulating Kinase 1. *Biol. Chem*. 276, 12749–12755.

Centre for Disease Control and Prevention. (2007) Rift Valley fever outbreak, Kenya, November 2006– January 2007. *Morb. Mortal Wkly. Rep.* 56, 73–86.

Centre for Disease Control and Prevention. (2004) Life stages of *Anopheles* mosquitoes. *Morb. Mortal Wkly. Rep.* 52, 989-997

Christophers, S.R. (1933) The fauna of British India including Ceylon and Burma. Diptera, Vol. IV. *Hyg & Trop. Med.*28, 1-206.

Clark, A.G. and Shamaan, N.A. (1984) Evidence that DDT-dehydrochlorinase from the house fly is a glutathione S-transferase. *Pestic. Biochem. Physiol*. 22, 249-261.

Clark, A.G., Shamaan, N.A., Sinclair, M.D. and Dauterman, W.C. (1986) Insecticide metabolism by multiple glutathione S-transferases in two strains of the house fly, *Musca domestica* (L). *Pestic. Biochem. Physiol.* 25, 169-175.

Cho, S.G., Lee, Y.H., Park, H.S., Ryoo, K., Kang, K.W., Park, J., Eom, S.J., Kim, M.J., Chang, T.S., Choi, S.Y., Shim, J., Kim, Y., Dong, M.S., Lee, M.J., Kim, S.G., Ichijo, H. and Choi

EJ (2001) Glutathione S-transferase Mu modulates the stress-activated signals by suppressing apoptosis signalregulating Kinase 1. *Biol. Chem.* 276, 12749–12755.

Chiang, F.M., and Sun, C.N. (1993) Glutathione transferase isozymes of diamondback moth larvae and their role in the degradation of some organophosphorus insecticides. *Pestic. Biochem. Physiol*. 45, 7-14.

Chelvanayagam, G., Parker, M.W. and Board, P.G. (2001) Fly fishing for GSTs: a unified nomenclature for mammalian and insect glutathione transferases. *Chem. Biol. Interact*. 133, 256-260.

CYS_REC. http://sun1.softberry.com/berry.phtml?topic= cys_rec&group=help &subgroup=propt. (14/11/2010).

D

Das, B.P., Rajagopal, R. and Akiyama, J. (1990) Pictorial key to the species of Indian Anopheline Mosquitoes. *J. Pure Appl. Zoo*. 2, 131-162.

Das, S.C. and Baruah, I. (1985) Incrimination of *Anopheles minimus* Theobald and *Anopheles balabacensis balabacensis* Baisas (*An. dirus*) as a malaria vectors in Mizoram. *Indian J. Malariol*. 22(1), 53-55.

Ding, Y., Ortelli, F., Hemingway, J., Ranson, H. and Rossiter, L.C. (2003) The *Anopheles gambiae* glutathione transferase supergene family: annotation, phylogeny and expression profiles. *Biochem. J*. 4, 1-16.

Ding, Y., Hawkes, H., Meredith, J., Eggleston, P., Hemingway, J. and Ranson, H. (2005) Characterization of the promoters of Epsilon glutathione transferases in the mosquito *Anopheles gambiae* and their response to oxidative stress *Biochem. J*. 387, 879–888.

Djadid, N.D., Barjesteh, H., Forouzesh, F. and Zakeri, S. (2008) Determination of glutathione S-transferase e2 region (*GSTe2*) in DDT susceptible and resistant *Anopheles stephensi* populations: significance and application of nucleotide and amino acid comparison. *Iranian J. Biotech*. 6, 92-97.

Dulhunty, A., Gage, P., Curtis, S., Chelvanayagam, G. and Board P. (2001) The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator. *J. Biol. Chem*. 276,3319–3323.

Dyar, H.G. (1922). The mosquitoes of the United States. *Proc. Unit. States Nat. Mus.* 62, 1-119.

Derraik, J.G.B. (2005) Mosquitoes breeding in container habitats in urban and peri-urban areas in the Auckland Region, New Zealand. *Entomotrop.* 20(2), 89-93.

Dabire, K.R., Diabate, A., Pare-Toe1, L., Rouamba1, J., Ouari1, A., Fontenille, D. and Baldet, T. (2008) Year to year and seasonal variations in vector bionomics and malaria transmission in a humid savannah village in west Burkina Faso. *J. Vector Ecol.* 33 (1), 70-75.

Devonshire A.L., Devine, G.J.&Moores, G.D. (1992) Comparison of micropate esterase assays and immunoassay fpr identifying insecticide resistant variants of *Myzus persicae* (Homoptera: Aphidae). *Entomol. Res.* 82, 459-464.

Dirr, H., Reinemer, P. and Huber, R. (1991) X-ray crystal structures of cytosolic glutathione S-transferases. Implications for protein architecture, substrate recognition and catalytic function. *Eur. J. Biochem*. 220(3), 645–661.

Dabire, K.R, Diabate, A., Pare-Toe, L., Rouamba, J. Ouari1, A., Fontenille, D. and Baldet, D. (2008) Year to year and seasonal variations in vector bionomics and malaria transmission in a humid savannah village in west Burkina Faso. *J. Vect. Ecol.* 33(1), 70-75.

Devi, P. and R.K. Jauhari, R.K. (2004) Altitudinal distribution of mosquitoes in mountainous area of Garhwal region : Part–I. *J. Vect. Borne Dis.* 41, 17–26.

Devi, P. and Jauhari, R.K. (2006) A relationship between *Anopheles fluviatilis* and *An. stephensi* (Diptera: Culicidae) catches and the prevalence of malaria cases at Kalsi area in Dehradun district (Uttaranchal). *Indian J. Med. Res.* 123, 151–158.

Devi, P. and Jauhari, R.K. (2007) Mosquito species associated within some western Himalayas phytogeographic zones in the Garhwal region of India. *J. Insect Sc.* 7(32), 1-10.

Devi, P. and Jauhari, R.K. (2008) Reappraisal on anopheline mosquitoes of Garhwal region, Uttarakhand, India. *J. Vect. Borne Dis.* 45, 112–123

De Silva, D. and Hemingway, J. (2002) Structural organization of the *estα3*[1] gene in a Colombian strain in *Culex quinquefasciatus* differ from that in Cuba. *Med. Vet. Entomol.* 16, 99-105.

E

Enayati, A.A., Ranson, H. and Hemingway, J. (2005) Mini Review: Insect glutathione transferases and insecticide resistance. *Insect. Mol. Biol.* 14, 3-8.

Economics & Statistics dept. Govt. of Mizoram. Report (2010) http://desmizo.nic.in/file/Rainfall-2010.pdf

Expasy's ProtParam. http://us.expasy.org/tools/ protparam.html (12/11/2012).

Edillo, F.E., Toure, Y.T., Lanzaro, G.C., Dolo, G. and Taylor, C.E. (2002) Spatial and habitat distribution of *Anopheles gambiae* and *Anopheles arabiensis* (Diptera: Culicidae) in Banambani Village, Mali. *J. Med. Entomol.* 39, 70-77.

F

Finney, D.J. (1971) Probit analysis. 3rd ed. London, Cambridge University Press.

Fournier, D,, Bride, M., Poirie, M., Berge, J.B. and Plapp, F,W, (1992) Insect glutathione S-transferases. Biochemical characteristics of the major forms from houseflies susceptible and resistant to insecticides. *J. Biol. Chem.* 267, 1840-1855.

Feyereisen, R. (2005) Insect cytochrome P^{450}. *Comp. Mol. Insect Sc.* 4, 1-77.

G

Gulsiri, C., Vararattanavech, A., Leelapat, P., Prapanthadara, L. and Ketterman, A.J. (2006) Characterization of *Anopheles dirus* Glutathione Transferase Epsilon 4. *Mal. J.* 217, 156-163.

Grant, D.F. and Hammock, B.D. (1992) Genetic and molecular evidence for a *trans*-acting regulatory locus controlling glutathione S-transferase-2 expression in *Aedes aegypti*. *Mol.Gen. Genet.* 234, 169-176.

Gillies, M.T. and M. Coetzee. (1987) A supplement to the Anophelinae of Africa South of the Sahara (Afrotropical Region), vol. 55. Publications of the South African Institute of Medical Research, Johannesburg, South Africa, 143pp.

Grant, D.F. and Matsumura, F. (1989) Glutathione S-transferase 1 and 2 in susceptible and insecticide resistant *Aedes aegypti*. *Pestic. Biochem. Physiol.* 33, 132-143.

Grant, D.F., Dietze, E.C. and Hammock, B.D. (1991) Glutathione S-transferas isozymes in *Aedes aegypti:* purification, characterization, and isozyme specific regulation. *Insect Biochem*. 4, 421-433.

Glick, J.I. (1992) Illustrated keys to the female *Anopheles* of Southwestern Asia and Egypt (Diptera; Culicidae) *Mosq. Systemtematics*. 24(2), 155-153.

Grillet, M. (2000) Factors associated with distribution of *Anopheles aquasalis* and *Anopheles oswaldoi* (Diptera: Culicidae) in a malarious area, northeastern Venezuela. *J. Med. Entomol*. 37, 231–238.

Ganesh, K.N., Urmila, J., Guilet, P. and Manga, L. (2003) Pyrethroid susceptibility and enzyme activity in two malarial vector; *Anopheles stephensi* (Liston) and *A. culicifacies* (Giles) in Mysore, India. *Indian J. Med. Res.* 117, 30-38.

H

Herath, P.R.J., Hemingway, J., Weerasinghe, I.S. and Jayawardena, K.G.I. (1987) The detection and characterization of malathion resistance in field populations of *Anopheles culicifacies* B in Sri Lanka. *Pestic. Biochem. Physiol.* 29, 157–162.

Harbach, R.E. (2004) The classification of genus *Anopheles (*Diptera: Culicidae): a working hypothesis of phylogenetical relationships. *Bull. Entomol. Res*. 95, 537-553.

Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249(22), 7130-7139.

Hayes, J.D. and Wolf, C.R. (1988) *Role of glutathione transferase in drug resistance. In: Sies H, Ketterer B (eds) Glutathione conjugation: Mechanisms and Biological Significance.* Academic Press, London, pp. 315-355.

Huang, H.S., Hu, N.T., Yao, Y.E., Wu, C.Y., Chiang, S.W. and Sun, C.N. (1998) Molecular cloning and heterologous expression of a glutathione Stransferase involved in insecticide resistance from the diamondback moth, *Plutella xylostella*. *Insect Biochem. Mol. Biol.* 28, 651-658.

Hornby, J. A., D. E. Moore, and T. W. Miller Jr. (1994) *Aedes albopictus* distribution, abundance, and colonization in Lee County, Florida and its effect on *Aedes aegypti. J. Am. Mosq. Control Assoc*. 10, 397-402

Hernandez, S., Lanz, H., Rodriguez, M.H., Gonzalez-Ceron, L. and Tsutsumi, V. (2002) Cellular-mediated reactions to foreign organisms inoculated into the hemocoel of *Anopheles albimanus* (Diptera: Culicidae) *J. Med. Entomol.* 39, 61–69.

Hemingway, J., Malcolm, C.A., Kissoon, K.E., Boddington, R.G., Curtis, C.F. and Hill, N. (1985) The biochemistry of insecticide resistance in *Anopheles sacharovi*: comparative studies with a range of insecticide susceptible and resistant *Anopheles* and *Culex* species. *Pestic. Biochem. Physiol.* 24, 68–76.

Hemingway, J., Miyamoto, J. and Herath, P.R.J. (1991) A possible novel link between organophosphorus and DDT insecticide resistance genes in *Anopheles*: supporting evidence from fenitrothion metabolism studies. *Pestic. Biochem. Physiol.* 39, 49-56.

Hemingway, J., Small, G.J., Monro, A., Sawyer, B.V. and Kasap, H. (1992) Insecticide resistance gene frequencies in *Anopheles sacharovi* populations of the Cukurova plain, Adana province, Turkey. *Med. Vet. Entomol.* 6, 342–348.

Hemingway, J. (2000) Mini review: The molecular basis of two contrasting metabolic mechanisms of insecticide resistance. *Insect Biochem. Mol. Biol.* 30, 1009-1015.

Hemingway, J. and Ranson, H. (2000) Insecticida resistance in insect vectors of human disease. *Annu. Rev. Entomol.* 45, 371-391.

Hemingway, J., Hawkes, N.J., McCarroll, L. and Ranson, H. (2004) The molecular basis of insecticide resistance in mosquitoes. *Insect. Biochem. Mol. Biol.* 34, 653-665.

Health dept. Govt. of Mizoram, Report. (2010) http://health.mizoram.gov.in/

Health dept. Govt. of Mizoram, Report. (2012) http://health.mizoram.gov.in/

Hassan, A., Narayanan, V. and Salmah, M. (1993) Observations on the physico-chemical factors of the breeding habitats of *Culex quinquefasciatus* Say, 1823 (Diptera: Culicidae) in towns of north western Peninsular Malaysia. *Ann. Med. Entomol*. 2, 1-5.

Hazelton, G.A. and Lang, C.A. (1983) Glutathione S-transferase activities in the yellow-fever mosquito [*Aedes aegypti* (Louisville)] during growth and aging. *Biochem. J.* 210, 281-287.

I

Iyengar, M.O.T. (1932) *Anopheles* breeding in relation to season. *Indian J. Med. Res.* 19, 917-939.

J

Jirajaroenrat, K., Pongjaroenkit, S., Krittanai, C., Prapanthadara, L. and Ketterman, A.J. (2001) Heterologous expression and characterization of alternatively spliced glutathione S-transferases from a single *Anopheles* gene. *Insect Biochem. Mol. Biol*. 31, 867-875.

Johansson, A.S. and Mannervik B. (2001) Human glutathione transferase A3-3, a highly efficient catalyst of doublebond isomerization in the biosynthetic pathway of steroid hormones. *J. Biol. Chem*. 276, 32061–32065.

Jose, G.B. and Derraik, G.B. (2005) Mosquitoes breeding in container habitats in urban and peri-urban areas in the Auckland Region, New Zealand. *Entomotropic*. 20(2), 89-93.

Jakoby, W.B. and Ziegler, D.M. (1990) The enzymes of detoxication. Mini review. *J. Biol. Chem.* 265, 20715-20718.

K

Kim, J.H., Dahms , H.U., Rhee, J.S., Lee, Y.M., Lee, J., Han , K.N. and Lee, J.S. (2010) Expression profiles of seven glutathione S-transferase (GSTs) genes in cadmium-exposed river pufferfish (*Takifugu obscurus*). *Com. Biochem. Physiol.* 151, 99–106.

Kostaropoulos, I., Papadopoulos, M., Metaxakis, A., Boukouvala, E. and Mourkidou, E. (2001) Glutathione Stransferase in the defence against pyrethroids in insects. *Insect Biochem. Mol. Biol.* 31, 313-319.

Kengluecha, A., Singhasivanon, P., Tiensuwan, M., Jones, M.J. and Sithiprasasna, R. (2005) Water quality and breeding habitats of Anopheline mosquito in Northwestern Thailand. *J. Trop.Med. Pub. Health*. 23(1), 36-43.

Kadous, A.A., Ghiasuddin, S.M., Matsummura, F., Scott, J. G. and Tanaka, K. (1983) Difference in the picrotoxinin receptor between the cyclodiene - resistant and susceptible strains of the German-cockroach. *Pesticide Biochem. Physiol.*19,157-166.

L

Listowsky, I., Abramovitz, M., Homma, H and Niitsu, Y. (1988) Intracellular binding and transport of hormones and xenobiotics by glutathione Stransferase. *Drug Metabol. Review*. 19, 305-318.

Li, X,, Schuler, M.A. and Berenbaum, M.R. (2007) Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu. Rev. Entomol.* 52, 231-253.

Lo Bello, M., Nuccetelli, M., Caccuri, A. M., Stella, L., Parker, M. W., Rossjohn, J., McKinstry, W. J., Mozzi, A. F., Federici G. and Polizio F., (2001) Human glutathione transferase P1-1 and nitric oxide carriers: A new role for an old enzyme. *J. Biol. Chem*. 276, 42138– 42145.

Lee, H.L. (1990) A rapid and simple biochemical method for the detection of insecticide resistance due to elevated esterase activity in *Culex quinquefasciatus*. *Tro. Biomed*. 7, 21–28

Limrat, D., Rojruthai, B., Apiwathnasorn, C., Samung, Y. and Prommongko, S. (2001) *Anopheles barbirostris/campestris* a probable vector of Malaria in Aranyaprathet, Sa Kaeo province. *Southeast Asian J Trop Med Public Health.* 32(4), 739-744.

Lumjuan, N. (2005) Characterisation of *Aedes aegypti* glutathione Stransferase enzyme family. Ph.D. Thesis, Liverpool School of Tropical Medicine, Liverpool, UK.

Lumjuan, N., McCarrol, L., Prapanthadara, L., Hemingway, J. and Ranson, H. (2005) Elevated activity of an Epsilon class glutathione transferase confers DDT resistance in the dengue vector, *Aedes aegypti. Insect Biochem. Mol. Biol*. 35, 861-871.

Lines, J.D. and Nassor, N.S. (1991) DDT resistance in *Anopheles gambiae* declines with mosquito age. *Med. Vet. Entomol*. 5, 261-265.

Listowsky, I., Abramovitz, M., Homma, H. and Niitsu, Y. (1988) Intracellular binding and transport of hormones and xenobiotics by glutathione Stransferase. *Drug Metab. Rev.* 19, 305-318.

Lipke, H. and Chalkley, J. (1962) Glutathione, oxidized and reduced, in some/ dipterans treated with 1,1,1-trichloro-2,2-di-(*p*-chlorophenyl) ethane. *Biochem. J.* 85, 104-109.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193(1), 265-275.

M

Malhotra, P.R., Sarkar, P.K. and Bhuyan, M. (1982). Mosquito survey in Nagaland. *Indian J. Publ. Hlth.* 26(3), 163-168.

Malhotra, P.R., Bhuyan, M. and Baruah, I. (1984). Mosquitoes of Mizoram. *J. Malariol.* 21, 126-126.

Malhotra. P.R (1994) Check-list of mosquitoes of Northeast India (Diptera; Culicidae). *Orient. Insects.* 28, 125-149.

Mendoza, A.C., Penilla, R.P. and Rodriguez, D.A. (2009) Insecticide resistance and glutathione S-transferases in mosquitoes: *African J. Biotech.* 8, 1386-1397.

Mannervik, B., Danielson, U. H. (1988) *Glutathione transferases – structure and catalytic activity.* CRC. *Crit. Rev. Biochem*. 23, 283–337.

Mogi, M., Sunahara, T. and Selemo, M. (1999) Mosquito and aquatic predator communities in ground pools on lands deforested for rice-field development in central Sulawesi, Indonesia. *J. Am. Mosq. Cont.r Assoc.* 15(2), 92–107.

Mwangangia, J.M., Ephantus, J. Muturib and Mbogoa C.M. (2009) Seasonal mosquito larval abundance and composition in Kibwezi, lower eastern Kenya .*J Vector Borne Dis.* 46, 65–71.

Mwangangia, J.M.,, Mbogoa, C.M., Muturic, J.E., Joseph, G., Nzovua, J.G., Githureb, J.I., and John C. (2007) Spatial distribution and habitat characterisation of *Anopheles* larvae along the Kenyan coast. *J. Vect. Borne Dis.* 44, 44–51.

Mogi, M. (1978) Population studies on mosquitoes in the rice field are of Nagasaki, Japan, especially on *Culex tritaeniorhynchus*. *Trop. Med*. 20, 173-263.

Muturi, J., Shililu, B., Jacob, J., Githure, W., Gu and Novak, R. (2006) Mosquito species diversity and abundance in relation to land use in a riceland agroecosystem in Mwea, Kenya. *J. Vector Ecol*. 31, 129-137.

Mannervik, B, and Danielson, U.H. (1988) Glutathione transferases—structure and catalytic activity. *Crit. Rev. Biochem.* 23, 283-337.

Mourya, D.T., Gokhale, M.D., Chakraborti, S., Mahadev, P.V. and Banerjee, K. (1993) Insecticide susceptibility status of certain populations of *Aedes aegypti* mosquito from rural areas of Maharashtra state. *Indian J. Med. Res.* **7**, 87-91.

Malcolm, C. A. and Wood, R. J. (1982) Location of a gene conferring resistance to knockdown by permcthrin and bioresmethrin in adults of the BKPM3 strain *of Aedes aegypti. Genetica.* 59, 233-237.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca G.A.B. and Kent, J. (2000) Biodiversity hotspots for conservation priorities. *Nature.* 403, 853-858.

Mathenge, E.M., Gimnig, J.E., Kolczak, M., Ombok, M., Irungu, L.W. and Hawley, W.A. (2001) Effect of permethrin-impregnated nets on exiting behaviour, blood feeding success, and time of feeding of malaria mosquitoes (Diptera: Culicidae) in Western Kenya. *J.Med. Entomol.* 38(4), 531–536.

Minakawa, N., Mutero, C.M., Githure, J.I., Beier, J.C. and Yan, G. (1999) Spatial distribution and habitat characterization of Anopheline mosquito larvae in Western Kenya. *American J. Tro. Med. and Hygiene.* 61, 1010-1016.

Michael, A.J. and Martens, W.J.M. (1995) The health impact of global climate changes: grasping with scenarios, predictive models and multiple uncertainties. *Ecosyst. Hlth.* 1, 23–33.

N

National Vector Borne Diseases Control Programme. Mizoram State report, 2010 (NVBDCP-Mizoram report, 2010). www.mohfw.nic.in/NRHM/Documents/NE_Reports/Mizoram_Report.pdf

National Vector Borne Diseases Control Programme. Governament of India report (2013) http://www.nvbdcp.gov.in/Doc/mal-situation-Jan13.pdf

Nagpal, B.N. and Sharma, V.P. (1987) Survey of mosquito fauna of Northeastern region of India. *Indian J. Malariol.* 24, 143-149.

Nagpal, B.N. and Sharma, V.P. (1995) *Indian Anophelines*. Oxford and IBH Publishing CO. Pvt. Ltd. New Delhi.

O

Oo, T.T., Storch, V. and Becker, N. (2005) Review of the Anopheline mosquitoes of Myanmar. *J. Vector Ecol.* 29, 21-40.

Okogun, G.R., J.C. Anosike, A.N. Okere, and B.E. Nwoke. (2005) Ecology of mosquitoes of Midwestern Nigeria. *J. Vec. Borne Dis*. 42, 1-8.

Okogun, R.A., Nwoke, E.B., Okere, A.N., Anosike, J.C. and Esekhegbe, A.C. (2003) Epidemiological implications of preferences of breeding sites of mosquito species in Midwestern Nigeria. *Ann. Agric. Environ. Med.* 10, 217-222.

Okogun, G.R.A. (2005) Life table Analysis of Anopheles malaria vectors: generational mortality as tool in mosquito vector abundance and control studies. *J. Vector Borne Dis*. 42, 45-53.

Overgaard, H.J., Tsuda, Y., Suwonkerd, W. and Takagi, M. (2002) Characteristics of *Anopheles minimus* (Diptera: Culicidae) larval habitats in northern Thailand. *Environ. Entomol.* 31(1), 134–141.

Oppenoorth, F.J., Van der Pas, L.J.T. and Houx, N.W.H. (1979) Glutathione Stransferases and hydrolytic activity in a tetrachlorvinphos-resistant strain of housefly and their influence on resistance. *Pestic. Biochem. Physiol*. 11, 176-188.

Ortelli, F., Rossiter, L.C., Vontas, .J, Ranson, H. and Hemingway, J. (2003) Heterologous expression of four glutathione transferase genes genetically linked to a major insecticide-resistance locus from the malaria vector *Anopheles gambiae*. *Biochem. J.* 373, 957-963.

P

Perera, M.D.B., Hemingway, J. and Karunaratne, S.H.P. (2008) Multiple insecticide resistance mechanisms involving metabolic changes and insensitive target sites selected in anopheline vectors of malaria in Sri Lanka. *Mal. J.* 7, 168-178.

Pramanik, M.K. and Raut, S.K. (2005) *Toxorhynchites splendens*, a potential biological control agent of the pest and vector mosquitoes occurring in *boro* rice-fields in West Bengal, India. *Environ. Ecol.* 23(3), 698–704.

Piyaratnea, M.K., F.P. Amerasinghe, P.H. Amerasighe, and F. Konradsen. (2005) Physico-chemical characteristics of *Anopheles culicifacies* and *Anopheles varuna* breeding water in a dry zone stream in Sri Lanka. *J. Vec. Borne Dis*. 42, 61–67.

Prapanthadara, L., Hemingway, J. and Ketterman, A.J. (1995) DDT-resistance in *Anopheles gambiae* Giles from Zanzibar Tanzania, based on increased DDT-dehydrochlorinase activity of glutathione S transferases. *Bull. Entomol. Res*. 85, 267-274.

Pickett, C.B. and Lu, A.Y. (1989) Glutathione S-transferases: gene structure, regulation, and biological function. *Annu. Rev. Biochem*. 58, 743-764.

Prapanthadara, L., Hemingway, J. and Ketterman, A.J. (1993) Partial purification and characterization of glutathione S-transferases involved in DDT resistance from the mosquito *Anopheles gambiae*. *Pestic. Biochem. Physiol.* 47, 119-133.

Prapanthadara, L., Koottathep, S., Promtet, N., Hemingway, J. and Ketterman, A.J. (1996) Purification and characterization of a major glutathione Stranferase from the mosquito *Anopheles dirus* (species B*). Insect Biochem. Mol. Biol*. 26, 277-285.

Penilla, R.P., Rodríguez, A.D., Hemingway, J., Torres, J.L., Solis, F. and Rodríguez, M.H. (2006) Changes in glutathione S-transferase levels in DDT resistant natural Mexican populations of *Anopheles albimanus* under different insecticide resistance management strategies. *Pestic. Biochem. Physiol*. 86, 63-71.

Protprop. http://www.mzu.edu.in/schools/biotechnology.html (14/11/2012).

ProQ (Protein Quality predictor server). http://www.sbc.su.se/~bjornw/ProQ/ProQ.html (15/11/2012).

R

Reuben, R., Tewari, S.C., Hiriyan, J. and and Akiyama, J.(1994) Illustrated keys to species *Culex* associated with Japanese Encephalitis in Southeast Asia (Diptera; Culicidae) *Mosq. Systematics.* 26(2), 75-96.

Ranson. H. and Hemingway, J. (2005) Mosquito glutathione transferases. Review. *Methods Enzymol*. 401, 226-241.

Ranson, H., Claudianos, C., Ortelli, F., Abgrall, C., Hemingway, J., Sharakhova, M.V., Unger, M.F., Collins, F.H. and Feyereisen, R. (2002) Evolution of supergene families associated with insecticide resistance. *Sc.* 298, 179-181.

Ranson, H., Rossiter, L., Ortelli, F., Jensen, B., Wang, X., Roth, C.W., Collins, F.H. and Hemingway, J. (2001) Identification of a novel class of insect glutathione S-transferases involved in DDT resistance in the malaria vector, *Anopheles gambiae*. *Biochem. J.* 359, 295-304.

Ranson, H., Collins, F.H. and Hemingway, J. (1998) The role of alternative mRNA splicing in generating heterogeneity within the *Anopheles gambiae* class I glutathione S-transferase family. *Proc. Natl. Acad. Sci*. 95, 14284-14289.

Rodriguez, A.D., Rodriguez, M.H., Meza, R.A., Hernandez, J.E., Rejmankova, E., Savage, H.M., Roberts, D.R., Pope, K.O. and Legters, L. (1993) Dynamics of population densities and vegetation associations of *Anopheles albimanus* larvae in a coastal area of southern Chiapas, Mexico. *J. Am. Mosq. Control Assoc.* 9, 46-58.

Rao, T.R., Dhanda ,V,, Bha,t H.R. and Kulkarni, S.M. (1973) A survey of haematophagous arthropods of Western Himalaya, Sikkim and hill districts of West Bengal: A general account. *Indian .J Med. Res*. 61, 1421–1461.

Rajnikant, Pandey, S.D. and Sharma, S,K. (1996) Role of biological agents for the control of mosquito breeding in rice fields. *Indian J. Malariol.* 33, 209-215.

Rajmankova, E., Savage, H.M., Rodriguez, M.H., Roberts, D.R. and Rejmanek, M. (1992) Aquatic vegetation as a basis of classification of *Anopheles albimanus* Weideman (Diptera: Culicidae) larval habitats. *Environ. Entomol.* 21, 598-603.

Rajagopalan, P.K., Chandrahas, .RK. and Paniker, K.N. (1979) Mosquito selection in Pattu and different adjacent localities in Thanjavur district (Tamil Nadu) with particular reference to *Anopheles culicifacies* Giles. *Indian J. Med. Research.* 69, 589-597.

Rahman, S.J., Wattal, B.L. and Sharma, M.I.D. (1973) Ecology of mosquitoes of Nilgiri Hills (Tamil Nadu) with particular reference to vectors of human diseases. *Indian J.Entomol.* 35, 228-246.

Ranson, H., Prapanthadara, L. and Hemingway, J. (1997) Cloning and characterization of two glutathione S-transferases from a DDT-resistant strain *of Anopheles gambiae. Biochem. J.* 324, 97-102.

Reisen, W.K., Fang, Y. and Martinez, V.M. (2006) Effects of temperature on the transmission of West Nile virus by *Culex tarsalis* (Diptera: Culicidae). *J. Med. Entomol.* 43, 309-317.

Rajput, K.B. and Singh, T.K. (1988) Vertical distribution of mosquitoes in Manipur. *Entomol.* 13 (3&4), 295–301.

Rueda, L.M., Patel, K.J. and Axtell, R.C. (1990) Efficacy of encapsuled *Coelomomyces giganteum* (Oomycetes: Lagenidiales) against *Culex quinquefasciatus* and *Aedes aegypti* larvae in artificial containers. *J. American Mosq. Cont. Assoc.* 6, 694-699.

Rydzanicz, K. and Lonc, E. (2003) Species composition and seasonal dynamics of mosquito larvae in the Wroclaw, Poland area. *J. Vec. Ecol.* 28(2), 255-266.

Russell, R. (1999) Constructed Wetlands and Mosquitoes: Health Hazards and Management Options and Australian Perspective. *Ecol. Engineering.* 12, 107-124.

Russell, P.F. and Rao, H.R. (1972) On habitat and association of species of Anopheline larvae in south-eastern Madras. *J. Mal. Inst. of India.* 3, 153-178.

Rasmol. http://openrasmol.org/ (15/11/2012).

Robert, V., Awono-Ambene, H.P. and Thioulouse, J. (1998) Ecology of larval mosquitoes, with special reference to *Anopheles arabiensis* (Diptera: Culicidae) in Market-Garden Wells in Urban Dakar, Senegal. *J. Med. Entomol.* 35, 948-955.

Rajagopal, R. (1979) Role of *Anopheles balabacensis* in the transmission of malaria in Assam. *J. Commun. Dis*. 10(2), 71-74.

S

Sahu, S.S., Parida, S.K., Sadanandane, C., Gunasekaran, K., Jambulingam, P. and Das, P.K. (1990) Breeding habitats of malaria vectors: *Anopheles fluviatilis, An. annularis* and *An. culicifacies* in Koraput district, Orissa. *Indian J. Malariol.* 27, 209-216.

Sen, A. K., John, V. M., Krishnan, K. S. and Rajagopaj, J. R. (1973) Studies on malaria transmission in Tirap district, Arunachal Pradesh. *J. Commun. Dis.* 5 (2), 98-110.

Sarkar, P. I., Rao, K. M., Nath, D. R., Bhuyan, M. and CHakraborty, B. C. (1984) Seasonal incidence of mosquito breeding in plant axils at Tezpur (Assam). *Indian J. Publ. Hlth.* 28(3), 152-158.

Savage, H.M., Rejmankova, E., Arredondo-Jimenez, J.I., Roberts, D.R. and Rodriguez, M.H. (1990) Limnological and botanical characterization of larval habitats for two primary malarial vectors, *Anopheles albimanus* and *An. pseudopunctipennis*, in coastal areas of Chiapas State, Mexico. *J. Am. Mosq. Control Assoc.* 6, 612-620.

Sivakumar, K., Balaji, S. and Gangaradhakrishnan. (2007) *In silico* characterization of antifreeze proteins using computational tools and servers. *J. Chem. Sc.* 119, 571–579.

Shililu, J., Ghebre, T., Seulu, F., Mengistu, S., Fekadu, H., Zerom, M., Ghebregziabiher, N., Sintsath, D., Bretas, G., Mbogo, C., Githere, J., Brantly, E., Novak, R. and Beier, J.C. (2003) Larval habitat diversity and ecology of anopheline larvae in Eritrea. *J. Med. Entomol.* 40, 921-929.

Sawicki, R., Singh, S.P., Mondal, A.K., Benes, H. and Zimniak, P. (2003) Cloning, expression and biochemical characterization of one Epsilon-class (GST-3) and ten Delta-class (GST-1) glutathione S-transferases from *Drosophila melanogaster*, and identification of additional nine members of the Epsilon class. *Biochem. J*. 370, 661-669.

Sheehan, D., Meade, G., Foley, V.M. and Dowd, C.A. (2001) Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem. J*. 360, 1-16.

Sayed, A.M., Godde, J., & Arn, H. (2000) A computer-controlled video system for real-time recording of insect flight in three dimensions. *J. Insect Behavior*. 13, 881-900.

Singh, S.P., Coronella, J.A., Benes, H., Cochrane, B.J. and Zimniak, P. (2001) Catalytic function of *Drosophila melanogaster* glutathione Stransferase DmGSTS1-1 (GST-2) in conjugation of lipid peroxidation end products. *Eur. J. Biochem.* 268, 2912-2923.

Scanlon, J.E. (1965) Distribution in altitude of mosquitoes in northern Thailand. *Mosq. News.* 25, 137–144.

Stone, B.F. and Brown, A.W.A. (1969) Mechanisms of resistance to fenthion in *Culex pipiens* fatigans Wied. *Bull. WHO.* 40(3), 401–408.

Sivakumar, K. (2006) Proteomics: from sequence to structure. *Adv. Bio.Tech*. 4(11), 18-23.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning: A laboratory manual, 2nd ed*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Simms, D., Cizdziel, P.E and Chomczynski, P. (1993) TRIzol™: a new reagent for optimal single-step isolation of RNA. *Focus.* 15, 99-103.

Senthil Kumar, N. , Tang, B., Chen, X., Tian, H. and Zhang, W. (2008) Molecular cloning, expression pattern and comparative analysis of chitin synthase gene B in *Spodoptera exigua. Com. Biochem. Physiol.* 149, 401-540.

SSCP-Seq - Prediction from Protein Sequence - EMBL-Heidelberg (Germany). http://coot.embl.de/SSCP//sscp_seq.html (13/11/2012).

Sattler, M.A., Mtasiwa, D., Kiama, M., Premji, Z., Tanner, M., Killeen, G.F. and Lengeler, C. (2005) Habitat characterization and spatial distribution of *Anopheles sp*. Mosquito larvae in Dar es Salaam (Tanzania) during an extended dry period. *Malaria J.* 4(4), 1475-1482.

Sunish, I., Reuben, R. and Rajendran, R. (2006) Natural survivorship of immature stages of *Culex vishnui* (Diptera: Culicidae) complex, vectors of Japanese Encephalitis Virus, in rice fields in Southern India*. J. Med. Entomol.* 43, 185-191.

Sen, A. K., John, V. M., Krishnan, K. S. and Rajagopaj, J. R. (1973) Studies on malaria transmission in Tirap district, Arunachal Pradesh. *J. Commun. Dis.* 5(2), 98-110.

Sarkar, P. I., Rao, K. M., Nath, D. R., Bhuyan, M. and CHakraborty, B. C. (1984) Seasonal incidence of mosquito breeding in plant axils at Tezpur (Assam). *Indian J. Publ. Hlth.* 28(3), 152-158.

T

Tiimub, B.M., Adu, B.K., and Obiri-Danso, K. (2012) Physico-chemical Assessment of Mosquito Breeding Sites from Selected Mining Communities at the Obuasi Municipality in Ghana. *J. Envir. Earth Sc.* 2(10), 123-135.

Tang, A.H. and Tu, C.P. (1994) Biochemical caracterization of *Drossophila* glutathione S transferase D1 and D2. *J. Biol. Chem.* 269, 27876- 27884.

Toung, Y.P.S., Hsieh, T.S. and Tu, C.P.D. (1990) Drosophila glutathione Stransferase 1-1 shares a region of sequence homology with the maize glutathione S-transferase III. *Proc. Natl. Acad. Sci.*87, 31-35.

Tanaka, K., Nakajima, M. and Kurihara, N. (1981) The mechanism of resistance to lindane and hexadeuterated lindane in the third yumenoshima strain of house fly. *Pestic. Biochem. Physiol*. 16, 149- 157.

Trivedy, R.K., Goel, P.K. and Trisal, C.L. (1987) *Practical methods in ecology and environmental science.* Environmental Publications, Veekay Press Karad, India.

.

U

Udomsinprasert, R. and Ketterman, A.J. (2002) Expression and characterization of a novel class of glutathione S-transferase from *Anopheles dirus*. *Insect Biochem. Mol. Biol*. 32, 425-433.

Udomsinprasert, R., Pongjaroenkit, S., Wongsantichon, J., Oakley, A.J., Prapanthadara, L., Wilce, M.C.J. and Ketterman, A.J. (2005) Identification, characterization and structure of a new Delta class glutathione transferase isoenzyme. *Biochem. J*. 388, 763-771.

Ugaki, M., Shono, T., and Fukami, J. (1985) Metabolism of fenitrothion by organophosphorus-resistant and susceptible house flies, *Musca domestica* L. *Pestic. Biochem. Physiol.* 23, 33-40.

V

Vontas, J.G., Small, G.J. and Hemingway, J. (2001) Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. *Biochem. J.* 357, 65-72.

Vontas. J.G., Small, G.J., Nikou, D.C., Ranson, H. and Hemingway, J. (2002) Purification, molecular cloning and heterologous expression of a glutathione S-transferase involved in insecticide resistance from the rice brown planthopper, *Nilaparvata lugens*. *Biochem. J.* 362, 329-337.

Vulule, J.M., Beach, R.F., Atieli, F.K., MacAllister, J.C., Brogdon, W.G., Roberts, J.M., Mwangi, R.W. and Hawley, W.A. (1999) Elevated oxidase and esterase levels associated

with permethrin tolerance in *Anopheles gambiae* from Kenya villages using permethrin impregnated bednets. *Med. Vet. Entomol.* 13, 239-244.

Vaughan, A., Hawkes, N. and Hemingway, J. (1997) Co-Amplification explains linkage disequilibrium of two mosquito esterase genes in insecticide resistant *Culex quinquefasciatus*. *Biochem. J.* 325, 359-365.

W

Wang, J., McCommas, S. and Syvanen, M. (1991) Molecular cloning of a glutathione S-transferase overproduced in an insecticide resistant strain of the house fly (*Musca domestica*). *Mol. Gen. Genet*. 227, 260- 266.

Wei, S.H., Clark, A.G. and Syvanen, M. (2001) Identification and cloning of a key insecticide-metabolizing glutathione S-transferase (MdGST-6A) from a hyper insecticide-resistant strain of the house-fly *Musca domestica*. Insect Biochem. *Mol. Biol.* 31, 1145-1153.

World Health Organization (WHO). (1975) *Manual on practical entomology in malaria vector bionomics and organization of antimalaria activities*. Part I and part II, Offset Publication No.13, Geneva.

World Health Organization (WHO). (1998) *Techniques to detect insecticide resistance mechanisms (field and laboratory manual).* Geneva. (WHO/CDS/CP/MAL/98.6)

World Health Organization (WHO). (2005) Guidelines for laboratory and field testing of Mosquito larvicides. pp. 41. (WHO/CDS/WHOPES/GCDPP/2005.13)

World malaria report. (2005) www.rollbackmalaria.org/wmr2005/html/2-1.htm.

World Health Organization. (1963) Insecticide resistance and vector control. *Technical Report Series, Geneva.* 265, 41-47.

World Health Organization. (1981) Instructions for determining the susceptibility or resistance of adult mosquitoes to organochlorine, organophosphate and carbamate insecticides-diagnostic test. (WHO/VBC.81.806).

X

Xu, Q., Liu, H., Zhang, L. and Liu, N. (2005) Resistance in the mosquito, *Culex quinquefasciatus*, and possible mechanisms for resistance. *Pest. Manag. Sci.* 61(11), 1096-1102.

Y

Yang, Y., Cheng, J.Z., Singhal, S.S., Saini, M., Pandya, U. and Awasthi, S. (2001) Role of glutathione S-transferases in protection against lipid peroxidation. *J. Biol.Chem*. 276, 19220-19230.

Yin, Z., Ivanov, V. N., Habelhah, H., Tew, K. and Ronai Z. (2000) Glutathione S-transferase p elicits protection against H2O2-induced cell death via coordinated regulation of stress kinases. *Cancer Res*. 60, 4053–4057.

Yu, S.J. (1996) Insect glutathione S-transferases. *Zool. Stud.* 35(1), 9- 19.

Yuri.F., Bogdanov., Sergei Y., Dadashev, and Grishaeva, T.M. (2003) *In silico* search for functionally similar proteins involved in meiosis and recombination in evolutionarily distant organisms. *Silico Boil*. 3, 15-32.

Z

Zhou, Z.H., Lan, H. and Syvanen, M. (1998) A complex glutathione transferase gene family in the housefly *Musca domestica*. *Mol. Gen.* 256, 187-194.

Zhao, T., Singhal, S. S., Piper, J. T., Cheng, J., Pandya, U., Clark-Wronski, J., Awasthi, S. and Awasthi, Y. C. (1999) The role of human glutathione S-transferases hGSTA1-1 and hGSTA2-2 in protection against oxidative stress. *Arch. Biochem. Biophy*. 367, 216–224.

Zou, S., Meadows, S., Sharp, L., Jan ,L.Y. and Jan, Y.N. (2000) Genome-wide study of aging and oxidative stress response in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci.* 97, 13726-13731.

Ziegler, R., Whyard, S., Downe, A.E.R., Wyatt, G.R. and Walker, V.K. (1987) General esterase, malathion carboxylesterase, and malathion resistance in *Culex tarsalis*. *Pestic. Biochem. Physiol.* 8, 279–285.

Zimmerman, R.H. (1992) Ecology of malaria vectors in the Americas and future direction. *Mem. Insti. Oswaldo Cruz* , 87, 371–383.

IX. APPENDIX

Appendix- I

1. **Preparation of chemicals/reagents for Dissolved Oxygen estimation:**

i). $MnSo_4$:

182of $MnSo_4$ was dissolved in distilled water, it was filtered and diluted to 500 ml.

ii). Alkaline Iodide reagent with azide:

350 g of KOH and 75 g of KI was dissolved in distilled water to make the volume 500 ml. 5 g of Sodium Azide was dissolved separately in another beaker. It was then mixed together.

iii). Standard Sodium thiosulphate solution (N/40/0.025N):

6.205 g of Sodium thiosulphate (4R Grade) was dissolved in freshly boiled and cooled distilled water to 1 litre. One pellet of NAOH was added as preservative.

iv). Starch indicator:

1 g of starch was dissolved in 200 ml distilled water. It was then boiled and a drops of toluene was added as preservative.

2. Preparation of chemicals/reagents for Chloride estimation:

i). Standard Silver Nitrate Titrant (0.0141 N):

2.395 g of $AgNo_3$ was dissolved in distilled water and diluted on 1 litre. It was stored in dark bottle.

ii). Potassium chromate indicator:

10 g of $k_2Cr_2O_7$ was dissolved in distilled water. Silver Nitrate solution was added to produce red precipitate. It was stood for overnight, filtered and diluted to 200 ml with distilled water.

3. Preparation of chemicals/reagents for Total Hardness estimation:

i). Standard EDTA Titrant (0.01 M):

3.723 g of disodium salt of EDTA was dissolved in distilled water to prepared one litre of titrant.

ii). Ammonia Buffer solution:

114 ml of concentrated NH_4OH was added to 13.5 g of NH_4Cl. The volume was made to 200 ml by distilled water.

iii). Eriochrome Black T indicator:

0.5 g of Eriochrome Black T indicator was dissolved in 80 % ethyl alcohol.

4. Preparation of chemicals/reagents for Phosphate estimation:

i). Ammonium molybdate strong acid solution:

5 g of ammonium molybdate was dissolved in 55 ml distilled water. 62 ml of conc. H_2SO_4 was added to 80 ml of distilled water. It was cooled and ammonium molybdate was added to it and diluted to one litre by distilled water.

ii). Stannous Chloride solution:

0.5 g of fresh $SnCl_2$.H2O was dissolved in 2 ml of conc. HCl. It was diluted to 20 ml of distilled water.

iii). Standard Phosphate solution:

4.388 g of dry anhydrous potassium hydrogen orthophosphate (K_2HPO_4) was dissolved in distilled water to made the volume 1 litre (stock solution). From the stock solution 1 ml was taken and diluted to 99 ml of distilled water i.e. 100 times dilution. This was standard PO_4 solution that contained 10 mg PO_4/ litre. (1 ml = 0.01 mg PO_4).

5. Preparation of chemicals/reagents for Alkalinity estimation:

i). 0.02 N H_2SO_4 solution:

To 2.8 ml of H_2SO_4 was dissolved in 1 litre of distilled water to obtained 0.01 N of H_2SO_4 solution (stock solution). It was again diluted to 200 ml of distilled water to obtained 0.02 N acid titrant.

ii). Phenopthalein indicator:

50% ethanol (ethyl alcohol) solution was prepared which consisted of 50ml ethanol and 50 ml water. 0.5 g of phenolphthalein was dissolved in 50% ethanol (ethyl alcohol) solution and stored in cool place.

iii). Methyl Orange indicator:

0 .01 g of the Methyl Orange powder dissolved into 100 ml of distilled water.

1. **Stock solution**

1M K_2HPO_4 (Potasium Phosphate, dibasic): 34.48 g of K_2HPO_4 dissolved in 150 ml sterile mili-Q water. Make final volume to 200 ml.

1M K_2HPO_4 (Potasium Phosphate, monobasic): 27.22 g of KH_2PO_4 dissolved in 150 ml sterile mili-Q water. Make final volume to 200 ml. Autoclaved and stored at 4^oC.

1. **Potasium Phosphate Buffers:**

1M; pH 7.2:

1M K_2HPO_4 – 71.1 ml

1M K_2HPO_4 – 28.3 ml. Autoclaved and stored at 4^oC

1M; pH 6.5:

1M KH_2PO_4 – 32.95 ml

1M KH_2PO_4 – 67.05 ml. Autoclaved and stored at 4^oC

2. **0.2 M Sodium Acetate Buffer pH 5.0:**

i). 0.2 M Sodium Acetate:

3.28 g of Sodium Acetate dissolved in 150 ml sterile mili-Q water and made the volume 200 ml with sterile mili-Q water.

ii). 0.2 M acetic acid:

2.31 ml Glacial acetic acid dissolved in sterile mili-Q water.

iii). 0.2 M Sodium Acetate Buffer pH 5.0:

0.23 M Sodium Acetate - 35.2 ml

0.2 M Acetic acid - 14.8 ml. Autoclaved and stored at 4^oC

3. 30 mM Napthyl Acetate (α or β):

0.2793 g Napthyl Acetate dissolved in 50 ml Acetone and stored at 4^oC.

4. 3% Hydrogen peroxide:

3 ml Hydrogen peroxide dissolved in 97 ml methanol and stored at 4^oC.

5. TMBZ (3,3',5,'-Tetramethyl benzidine solution):

0.01 g TMBZ dissolved in 5 ml methanol and stored at 4^{o}C.

6. 5% SDS:

5 g SDS dissolved in 100 ml sterile water, membrane- filtered and stored at room temperature.

7. Reagent A: 2% Sodium carbonate in 0.1 NaOH:

0.4 g NaOH pellets dissolved in sterile water. To this 2 g of Na_2CO_3 was added and the final volume made upto 100 ml with sterile water.

8. 1% Copper sulphate:

0.01 g $CuSO_4$ dissolved in 1 ml sterile water.

9. 2% Potassium sodium tartarate:

0.02 g Potassium sodium tartarate dissolved in 1 ml sterile water.

10. Reagent B:

Reagent A – 100 ml

1% Copper sulphate – 1 ml

2% Potassium sodium tartarate – 1 ml, stored at 4^{o}C.

11. Reagent C:

Folin phenol colchicines reagent – 20 ml

Sterile water – 40 ml, stored at 4^{o}C.

12. **Protein standard (200 µg.ml):** 20 mg BSA (Bovine Serum Albumin) dissolved in 100 ml sterile water.

13. **Standard α-napthol (200 µg/ml):** 20 mg α-napthol dissolved in 0.02 M Phosphate buffers pH 7.2.

14. **Standard β-napthol (200 µg/ml):** 20 mg β -napthol dissolved in 0.02 M Phosphate buffers pH 7.2.

11. Working solutions:

1. Phosphate Buffers:

a). 0.02 M pH 7.2 (200 ml):

1M Phosphate buffers pH 7.2 – 4 ml

Sterile water – 196 ml

b). 0.625 pH 7.2 (200 ml):

1M Phosphate buffers pH 7.2 – 125 ml

Sterile water – 75 ml

c). 0.1 M pH 6.5 (200 ml):

1M Phosphate buffers pH 6.5 – 20 ml

Sterile water – 180 ml

2. 0.06 mM Napthyl acetate:

30mM stock (α or β) - µl

Sterile water – 990 µl

3. Fast blue stain:

Fast blue salts – 0.006 g

Sterile water – 600 µl

4. **10mM Reduced Glutathione (GSH):**
   GSH – 0.0081 g
   1M Phosphate buffers pH 6.5 – 2.5 ml

5. **63mM Chloronitrobenzene (CDNB):**
   CDNB – 0.013 g
   Methanol – 1000 µl

6. **CDNB – GSH solution:**
   63mM Chloronitrobenzene (CDNB) – 125 µl
   10mM Reduced Glutathione (GSH) – 2500 µl

7. **TMBZ- Sodium Acetate Buffer solution:**
   TMBZ – 1500 µl
   0.2 M Sodium Acetate buffer pH 5.0 – 4500 µl

Published papers and Communicated:

H. Vanlaltana, **K. Vanlalhruaia**, T. Lalruatkima, R. Lalthakima, P. C. Chhunthangpuia, Esther Lalhmingliani and H. T. Lalremsanga (2007) **Studies on the plankton community of Rih Dil, Myanmar**. *Science Vision* 7(4), 144-151.

Vanlalhruaia, N. Senthil Kumar and G. Gurusubramanian (2011) ***Bacillus sphaericus*** **in the biological control of mosquito vector complex**. *Science Vision* 11 (2), 61-71.

K. Vanlalhruaia, G. Gurusubramanian and N. Senthil Kumar (2013) **Habitats and species composition of mosquitos in Mizoram, India.** Paper communicated to Parasitology Research Journal.

K. Vanlalhruaia, G. Gurusubramanian and N. Senthil Kumar (2013) ***In-silico*** **characterization of GSTe-4 proteins of Mosquitoes.** Paper communicated to Interdisciplinary Sciences: Computational Life Sciences Journal.

Papers Presentation at Seminars / Conferences:

Presented paper entitled '**Diversity and Habitats characteristics of mosquitos in Mizoram**' in the **National seminar on Emerging Trends in Biosciences and Future Prospect** (November 29th -30th, 2011) organized by Department of Zoology, Pachhunga University College (a constituent college of Mizoram University), Aizawl, Mizoram.

Presented paper entitled '**Habitats and influences of environmental factors on the abundance of mosquitos in Mizoram**' in the **National seminar on Environment, Biodiversity, Veda and Traditional sytem** (April 10th -12th, 2012) organized by Department of Zoology, Mizoram University, Aizawl, Mizoram.

Tranning/Workshop participated:

Tranning course on **'Bioinformatics- General concept and Applications'** (March 26th to 27th, 2009) organized by the Bioinformatics Infrastructure Facility,Department of Biotechnology, Mizoram University, Aizawl, Mizoram.

Winter School on **'Tranning in Insect Taxonomy (DST-FIST Programme)'** (January 25th to 6th February, 2010) organized by Department of Zoology, Tripura University, Agartala, Tripura.

Workshop on **'Molecular Phylogenetics and Evolution'**(November 22nd to 24th, 2010) organized by Department of Biotechnology, Mizoram University, Aizawl, Mizoram.

Workshop on **'Random Amplified Polymorphic DNA (RAPD) Marker and its Application'** (May 20th to 21st, 2011) organized by Department of Zoology and Biotechnology, Mizoram University, Aizawl, Mizoram.

Tranning on **'1st Summer School cum Workshop'** (May 23rd to 27th, 2011) organized by Institutional Biotech Hub, Department of Pharmacy, RIPAN, Aizawl, Mizoram.

Tranning on **'Induction Tranning Programme for Vector Borne Diseases Consultants'** (25th June to 3rd August, 2012) organized by National Institute of Malaria Research, Sector-8, Dwarka, New Delhi.

Characterization and expression profiles of Glutathione - S- transferase (GSTs) gene in *Anopheles* mosquito vector complex

ABSTRACT

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ABSTRACT

The thesis incorporates "Characterization and expression profiles of Glutathione - S-transferase (GSTs) gene in *Anopheles* mosquito vector complex". Mosquito (Family: Culicidae) and mosquito-borne disease have been threatening human and animals. These haematophagous mosquitoes are of considerable to be medical and veterinary importance, because they transmitting various pathogens like bacterial (plaque, typhus, Lyme disease etc), viral (dengue, chikungunya, West Nile viral diseases, Japanese Encephalitis etc) and protozoan (malaria, kala-azar etc). Mosquito-borne parasitic diseases are endemic in many areas of the world, causing more than 3.2 billion people to be at risk (WHO, 1998) and the current outbreak of infectious diseases throughout India is still spreading extensively and affects about 1/3 of the human populations (Hemingway *et al*., 2004).

Mizoram (92.15-93.29^{o} E and 21.58-24.35^{o} N) is one of the Seven Sister States (Malaria endemic area) listed as in North Eastern India, sharing borders with the states of Tripura, Assam, Manipur and with the neighboring countries of Bangladesh and Burma. In India, Mizoram alone contributed 5.73% of deaths due to malaria in 2007 and 10.44% in 2010 (NVBDCP, 2013). Malaria being the main cause of death in Mizoram and 119 persons died of malaria with 79 males and 40 females during the year 2009 (Health dept., Mizoram report, 2010). The undertaking of MSVBDCP (Mizoram State Vector Borne Diseases Control Programme) mainly focused on prevention of death and morbidity due to malaria by human treatment, vector control by means of spraying insecticide (DDT): which was started since 1948 and personal protection by distributing LLINs (long lasting insecticidal nets); thereby neglecting effective and efficient surveillance system: entomological component

*viz.*mosquito habitats and seasonal abundance and their role in diseases transmission as well as the tolerance of insecticides against diseases vectors as the continuous and indiscriminate use of insecticide in a population will lead to the development of physiological resistance in the insects (Ganesh *et al*., 2003). Understanding the relationship between habitats, environmental factors, distribution and relative abundance of vectors in the targeted areas is essential to know the status of the complexity of the resistance segregating in field populations for an efficient application of mosquito control methods as well as malarial cases reduction (Perera *et al*., 2008).Therefore, major objectives of the present study were as follows:

1. To study the prevalence and abundance of mosquito species and their breeding habitats
2. To establish the baseline susceptibility status against a commonly used synthetic insecticides
3. Quantitative estimation of resistance enzymes (GST and esterases)
4. To study the expression of GST gene (resistant gene) and
5. Comparison of GSTs genes and isoforms in insects in relation to characterization and structure prediction of proteins using Bioinformatics tools.

I. Diversity, relative abundances and habitats characteristics of mosquito

The present study detailed on survey of mosquito of Mizoram and their relationship with its occurrence and their breeding habitats. Taxonomic key was prepared for the identification of adults mosquito. A total of 8328 mosquitoes collection representing the 5 genera of: *Anopheles*, *Aedes*, *Culiseta*, *Culex* and *Toxorhynchites* altogether of 20 species were collected. The most dominant genus was *Anopheles* followed by *Culex*,

Culiseta, *Aedes* and *Toxorhynchites.* Distribution and diversity studies showed the following pattern as *Culex quinquefasciatus* (39.67%), *Anopheles barbirostris* (29.50%), *Anopheles vagus* (14.51%), *Aedes albopictus* (3.62%), *Culex tritaeniorhynchus* (3.03%), *Culex mimeticus* (2.78%), *Culex bitaeniorhyunchus* (1.94%), *Culex tarsalis* (1.02%), *Culiseta melanura* (0.80%), *Culex peus* (0.82%), *Toxorhynchites splenden* (0.64%), *Anopheles philipinensis* (0.48%), *Culiseta inornata* (0.33%), *Anopheles jamesi* (0.29%), *Anopheles nivipes* (0.19%), *Anopheles willmori* (0.14%), *Anopheles jeyporiensis* (0.10%), *Anopheles minimus* (0.07%), *Anopheles dirus* (0.03%) and *Aedes aegypty* (0.02%). *Culex quinquefasciatus* was a dominant species and found throughout year followed by *Anopheles barbirostris*. The present study found the prevalence of *Culiseta melanura* and *Culiseta inornata* which were the first report in Mizoram

Relative abundance studies in different districts of Mizoram suggested that: *Culex quiquefasciatus* was mostly found throughout year and regarded as constant (C=80.1-100%). Two species (*Anopheles barbirostris* and *Anopheles vagus*) were frequent in most of the months (60.1 -80%) while four species: *Aedes albopictus, Anopheles philipinensis, Anopheles jamesi, Toxorhynchites splenden* were considered as infrequent (20.1-40%). The remaining species: *Culex tritaeniorhynchus, Culex mimeticus, Culex bitaeniorhyunchus, Culex tarsalis, Culiseta melanura, Culex peus, Culiseta inornata, Anopheles nivipes*, *Anopheles willmori, Anopheles jeyporiensis, Anopheles minimus, Anopheles dirus* and *Aedes aegypty* were less frequency and regarded as sporadic (C=0-20%). According to the density criterion, three species (*Culex quiquefasciatus, Anopheles barbirostris, Anopheles vagus*) are within the dominant class (D>5%); one of them,

Culex quiquefasciatus, also showed the highest constancy during the study peroid. Five species (*Culex mimeticus, Culex tritaeniorhynchus, Culex tritaeniorhynchus, Culex bitaeniorhyunchus* and *Aedes albopictus*) were included in the subdominant class (1< D <5%) while twelve species: *Culex tarsalis, Culiseta melanura, Culex peus, Culiseta inornata, Anopheles nivipes*, *Anopheles willmori, Anopheles jeyporiensis, Anopheles minimus,Anopheles dirus* and *Aedes Aegypty, Anopheles jamesi* and *Toxorhynchites splenden,* were less dominant and regarded as satellite species (D < 1%).

The survey found the presence of mosquito vector complexes in Mizoram, as there may be a chance of other vector borne diseases transmission in Mizoram (the neighboring states were already infected by these diseases) as dengue infection started in Mizoram since 2012. *Anopheles dirus* (0.03%) and *Anopheles minimus* (0.07%), a primary vector of Malaria (Das and Baruah, 1985), were found in less numbers while *Anopheles barbirostris* (29.50%): a incriminated malaria vector was predominant species and even found in malaria endemic study area. Therefore, there may be a chance of vector transmission.

Seasonal-wise mosquitoes variation and relative abundance studies in Aizawl district shows that from the overall collection of mosquito individual (n=4948), it was found that *Anopheles barbirostris* (29.20%) was the dominant species, followed by *Culex quinquefasciatus* (27.49%), *Anopheles vagus* (21.40%), *Culex tritaeniorhynchus* (5.11%), *Culex mimeticus* (4.67%), *Aedes albopictus* (4.02%), *Culex bitaeniorhyunchus* (3.27%), *Culex tarsalis* (1.71%), *Culex peus* (1.37%), *Toxorhynchites splenden.* (0.57%), *Anopheles philipinensis* (0.56%), *Culiseta melanura* (0.28%) and *Anopheles willmori*

(0.12%). Three years survey (2009-2011) data revealed that between the season, the total number of mosquito vary significantly ($P<0.001$), variation in mosquito species was also significant ($P<0.05$). The diversity index (H') and evenness aspect of diversity (H_{eve}) analysis showed that mosquito diversity was highest in Monsoon season (Jul-Sep).The Tukey's test revealed significant differences in abundance of various species of mosquitoes ($P = 0.0001$). *Anopheles barbirostris* was predominant species and its abundance was significantly different from other mosquito species.

Based upon habitats preferences, collected mosquitoes were distinguished into four groups according to the coefficient of association obtained. Group-I includes 8 species: *Culiseta melanura, Culex tarsalis, Culex peus, Aedes albopictus, Culex tritaeniorhynchus, Culex bitaeniorhyunchus, Culex mimeticus and Culiseta inornata,* Group-II includes 6 species: *and Anopheles barbirostris, Anopheles vagus, Culex quinquefasciatus, Anopheles jamesii, Anopheles philipinensis* and *Anopheles* nivipes. Group-III includes 3 species: *Anopheles jeyporiensi, Anophelesmimimus* and *Anopheles willmori.* Group-IV includes 2 species: *Aedes aegypty* and *Toxorhynchites splenden.*The highest association coefficient (0.96) was found between *Culex tarsalis* and *Culex peus*, followed by *Culiseta melanura* and *Culex tritaeniorhynchus* (0.95). Since, the lowest coefficient of association (0.25) was found between *Culex mimeticus* and *Aedes aegypty* which fall under different group. The breeding habitats of mosquito species found in the present study were seepage pools, riverbeds, ponds, tanks, ditches, streams, rock holes, tree holes, intradomestic containers and shallow pits. Breeding habitat such as ponds and ditches were the richest habitats sharing 15 different mosquito species while the lowest

breeding habitats of mosquito species (3 species) was recorded from tanks. Natural habitats of temporary conditions with shady to partially shady and moderate vegetation were the main habitats preferences of collected mosquito species.

Studies on relationship between mosquito larval abundance and environmental variables resulted that: pH, Dissolved Oxygen, Alkalinity, Temperature, Total Dissolved Solid and Hardness of water were the main biotic factors that influenced the breeding of mosquito species. *Anopheles barbirostris* was positively associated with pH ($P<0.05$). High alkalinity in the habitat was positively associated with abundance of *Anopheles vagus* ($p<0.05$) while there was a negative association against dissolved oxygen ($p<0.01$). Habitats with alkaline water bodies and slightly turbid with amount of detritus has a positive association against *Culex quinquefasciatus* ($P<0.05$) but *Anopheles philipinensis* larval abundance was negatively associated with temperature ($P<0.05$) and found mostly in small, open, clear water habitats thus, positively associated with hardness of water and dissolved oxygen ($P<0.05$). There was a positively associated between *Culex tritaeniorhynchus* and alkalinity of water ($P<0.05$).

II. Insecticides susceptibility status of mosquitoes

Based upon susceptibility tests on the field collected population, *Anopheles vagus* (LC_{50}=1.340) was more susceptible against DDT than *Anopheles barbirostris* ((LC_{50}=1.607) while in case of deltamethrin susceptibility tests, *Anopheles vagus* (LC_{50}=0.0063) was found 1.23 fold increased tolerace level against deltamethrin as

compared to *Anopheles barbirostris* (LC_{50}=0.0051). Thus, *Anopheles barbirostris* was able to tolerate more against DDT than *Anopheles vagus* while the level of tolerance against deltamethrin was higher in *Anopheles vagus.* Therefore, it was found that based upon susceptibility tests there was a possibility of resistant on DDT (WHO, 1998) in *Anopheles barbirostris* and *Anopheles vagus* that needs to confirm.

III. Biochemical assay (GST and enterase) on insecticides treated mosquito samples

Biochemical assays resulted that, In DDT treated samples, the amount of GST enzyme production was highest in *Anopheles barbirostris* (0.420±0.02) and there was a correlation between increasing concentrations of DDT and enzyme elevation ($r = 0.953$; $P<0.05$). A similar pattern was also found in esterase assays that, esterase elevation was found correlated with increasing concentrations of DDT. In deltamethrin treated samples, GST enzyme production was higher in *Anopheles barbirostris* (0.320±0.02) than *Anopheles vagus* (0.253±0.02). There was a significant correlation of GST enzyme elevation and increasing concentrations of deltamethrin ($P<0.05$) while the level of esterases production was higher in *Anopheles vagus* (α- 0.035±0.01; β- 0.041±0.01) than *Anopheles barbirostris* (α- 0.021±0.01; β- 0.034±0.01) but there was no significant correlation between the level of esterases elevation and increasing deltamethrin concentrations. The insecticidal bioassay and biochemical assays results showed that the level of tolerant against DDT was higher in *Anopheles barbirostris* than *Anopheles vagus* and elevation of resistant enzymes (GSTs and esterases) production in both species were significanty correlated with increasing insecticides concentrations; but both species were

susceptible to deltamethrin even there was no significant correlation of the biochemical assays. Therefore, it was found that based upon susceptibility tests there was a possibility of resistant on DDT (WHO, 1998) in *Anopheles barbirostris* and *Anopheles vagus* that needs to confirm.

IV. Expression of GSTe4 gene in Anopheles species

The study of mRNA expression profile and *in-vitro* expression of GST gene in two *Anopheles* specimens (*Anopheles barbirostris* and *Anopheles vagus*) collected from the Aizawl district revealed 550 bp fragments of GSTe4 gene on 1.5% (w/v) agarose gel. Therefore, *Anopheles barbirostris* and *Anopheles vagus* were characterized as less susceptible against DDT and there was possibility of resistance that needs to confirm (WHO, 1998).

V. *In-silico* characterization of mosquito GST

Computational tools and online server characterized mosquito GSTe4 proteins as hydrophobic in nature that may be due to presences of high non-polar residues. Computed GSTe4 proteins were mostly basic in nature, can be analyzed using UV spectral methods. They were stable for a wide range of temperature and having a properties of better interaction with water. The SS bonds predicted from the primary structure (protein sequence) using CYS_REC tool might not be correct and the SS bonds identified from the three-dimensional structure (3D coordinates) using the Rasmol tool

might be correct. According to ProQ validation, the modelled 3D structures were characterized as fairly good model based on Maxsub score and extremely good model based on LG score.