Training Manual on Malaria Entomology

For Entomology and Vector Control Technicians (Basic Level)

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Training Manual on Malaria Entomology

For Entomology and Vector Control Technicians (Basic Level)

Integrated Vector Management of Malaria and Other Infectious Diseases Task Order 2
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Introduction

Malaria remains a major cause of death and sickness in most tropical regions of the world, where it is endemic in 106 countries. In 2010, about 81% of the total 216 million malaria cases took place in Africa, and 13% of cases occurred in Southeast Asia[1]. The largest proportion (91%) of the estimated 665,000 annual deaths from malaria occurs in Africa, mainly affecting children under five years old (86%). In the region of the Americas, over 670,000 confirmed malaria cases occurred in 2010, with 133 malaria-attributed deaths. Transmission is active in 21 countries putting about 20% of the population of the Americas at risk. Malaria imposes severe constraints on economic development and is an important cause of poverty in most disease endemic countries.

Although there has been sharp increase in funding for malaria control, disease reduction targets set by Roll Back Malaria[2] and National Malaria Control Programs still remain unachieved in many countries. This is partly due to the lack of capacity in generating adequate knowledge on local disease epidemiology to inform cost-effective implementation and program management. In particular, capacity for entomological monitoring and surveillance is still rudimentary in many endemic countries. There is an urgent need for National Malaria Control Programs to create adequate numbers of trained personnel to effectively participate in malaria control activities and support generation.

Purpose of the manual

A two-tier training course for entomology technicians has been developed aimed at facilitating the strengthening of core competencies for entomological monitoring and surveillance in disease endemic countries. This manual is aimed at guiding a basic level (Tier-1) entomology course, covering:

1. The life-cycle and bionomics of mosquitoes;
2. Adult and larval sampling, mosquito identification and incrimination of malaria vectors;
3. Primary malaria transmission indices and what they represent;
4. Malaria vector control and current primary interventions;
5. The role of entomology in vector control;
6. Basic principles for laboratory rearing of mosquitoes;
7. Tests of mosquito susceptibility and residual efficacy of insecticides used in vector control.

[1] WHO (2009). World Malaria Report 2011. World health Organization. Geneva, Switzerland (http://www.who.int/malaria/world_malaria_report_2011/en/)
[2] Roll Back Malaria – Global Malaria Partnership (http://www.rbm.who.int/index.html)

This manual presents the basic issues that are critical to understanding the primary educational goal of each subject area. It is however anticipated that the training course will provide extended opportunities for field work to assure a fuller learning experience and consolidate practical skills/competencies. Adult learning and participatory format is anticipated, in which students should be encouraged to find out things for themselves and from each other. A sample of the curriculum and schedule for the basic entomology technician course can be found in Annex I.

Target audience of the manual

This basic manual targets district level personnel in malaria endemic countries who will normally form the cadres that collect and report on local entomological indicators in support of vector control programs. They will normally have secondary level education or diploma in a subject area that lends itself to training in entomology.

Further readings

This manual was prepared based on several existing guidelines, manuals and published articles, including:

1. Benedict M (2009). *Methods in Anopheles research*. Malaria Research and Reference Reagent Center. Version 3. 264 pp
2. Hay SI, Sinka ME, Okara RM, Kabaria CW, Mbithi PM, Tago CC, Benz D, Gething, PW, Howes RE, Patil AP, Temperley WH, Bangs MJ, Chareonviriyaphap T, Elyazar IR, Harbach RE, Hemingway J, Manguin S, Mbogo CM, Rubio-Palis Y, Godfray HC (2010) Developing global maps of the dominant *Anopheles* vectors of human malaria. *PLoS Medicine* 7: e1000209.
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List of Useful Terms

Aestivation: A state of dormancy and inactivity that organisms undergo to survive extreme high temperatures and arid conditions imposed by the hot season.

Anthropogenic: Refers to any effect that relates to, or results from, the impact of human activity in nature.

Antibodies: These are particular proteins (called immunoglobulins) that are used by the immune system to recognize and neutralize foreign substances inside the body, such as virus, bacteria or parasites.

Biological species: A group of populations, or organisms in a population, that actually or potentially interbreed in nature and produce fertile offspring.

Bionomics: When referring to mosquitoes, this term translates into the biological, ecological and behavioral characteristics of a species or population that influence its capacity to act as a vector of disease.

Cytogenetics: Study of the structure and function of chromosomes (inherited structures that carry the genes that determine sex and the characteristics of an organism). When cytogenetics is applied to the identification and study of the relations between biological species, it is referred to as **cytotaxonomy**.

Epidemiology: Study of the distribution, patterns and determinants of health-characteristics and health-events (such as diseases) in populations, and how it is applied to control disease and health problems.

Genetic material: This is the biological material that is present in all living organisms and can be passed on from one generation to the next (inheritable). The genetic material determines the structure and function of the cells that form an organism. A **gene** is a particular sequence of the genetic material that determines a specific protein. In the cell, the genetic materials are organized in structures called **chromosomes**.

Genetic mutation: This term refers to any change in the DNA nucleotide sequence of a given gene. These changes may result in modifications of the protein that the gene codes for. Organisms carrying the mutation are called **mutants**, in opposition to wild-type individuals.

Morphology: This term refers to the size, shape and structure of organisms, or of the body parts that constitute them (both internal and external). The term **anatomy** is often used in place of morphology, in that it studies the organization and structure of organisms.

Parity: The number of times that a female has given birth to an offspring. A mosquito female that has laid eggs at least once in her life is called parous. Females that have not yet laid eggs are called nulliparous.

Polytene chromosome: These are giant chromosomes formed by multiple copies of the genetic material of which they are composed. These giant chromosomes occur only in certain cells of insects. Due to their size, polytene chromosomes are very useful for cytogenetic analysis.

Protein: These are biochemical compounds (molecules) that make up the cells of living organisms. Proteins play a role in all biological functions.

Sexual dimorphism: Characteristic differences between males and females of the same species.

Spiracles: Circular openings in the body of insects that allow air to enter inside the body.

Sporogonic cycle: This is the part of the malaria parasite life-cycle that develops inside the mosquito. It starts when the mosquito takes a blood meal from an infected human (or other vertebrate) host. Inside the mosquito stomach, the sexual parasite stages (gametocyte) come together to form an egg (ookinete) that moves to the stomach cell wall. The ookinete develops into an oocyst in the external face of the stomach wall. Inside the oocyst, sporozoites are formed by a process of cell division (meiosis). The oocyst then bursts, releasing the sporozoites that then move to the salivary glands, invading them. Once in the salivary glands, sporozoites will be passed to a human host the next time the mosquitoes bite and blood feed. There are some antimalarial drugs that target specific parasite stages during the sporogonic cycle. Gametocytocidal drugs kill gametocytes and sporontocidal drugs kill sporozoites. An example of such drugs is Primaquine.

Sporozoite: This is the life-stage of the malaria parasite in the mosquito that is capable of producing infection in humans (or other vertebrate hosts). Thus, it is the stage of the malaria parasite that is **infective** to humans. Sporozoites are found in the salivary glands of the mosquitoes. The outer cell wall of the sporozoite is covered by a stage-specific protein called **circumsporozoite** protein. This protein is what is tested for in the laboratory to determine if a mosquito is infective (will transmit malaria parasite when it bites) or not.

Unit 1
Malaria Control and the Role of Entomology

Learning Objectives

This unit is aimed at providing basic knowledge on:

- **Current measures used to control malaria.**
- **The major vector control measures.**
- **The role of entomology in the control of malaria.**
- **Important factors to consider when planning malaria vector control.**

1.1 Major components of malaria control programs

Malaria control programs normally include three basic components:

- Early detection and effective treatment of malaria cases
- Control of the mosquito vector(s)
- Community education

Early diagnosis and effective treatment of patients (chemotherapy)

The use of antimalarial drugs is the main tool available for reducing parasite populations. Besides treatment and prophylaxis, gametocytocidal and sporontocidal antimalarials affect the sporogonic development in the mosquito and thus malaria transmission.

Currently, most malaria control programs have adopted strategies of Early Case Detection and Prompt Treatment of malaria cases. These strategies imply the implementation of drug distribution centers and rapid diagnostic posts at the primary health system level. To be most effective, people at risk of getting malaria need to know the symptoms of malaria and be ready to seek proper treatment. The placement of trained persons within the community (*e.g.* community health workers) to help identify malaria and facilitate access to effective treatment has been found to be critical in patients receiving prompt treatment.

However, several problems undermine these methods. Obstacles regarding drug accessibility are a common reality. In addition, there are problems of poor compliance, related to economic reasons and to the side-effects of some drugs. As a result, therapeutic regimes are often not completed, increasing the risk of drug resistance. The rise and spread of resistance to antimalarial drugs such as chloroquine and pyrimethamine/sulfadoxine (Fansidar®) is a major obstacle to the sustainability of the parasitological component of malaria control programs.

Vector control and principles for effective implementation

Vector control is a major element of the Global Malaria Control Strategy of the World Health Organization. It remains the most effective way to prevent malaria transmission. Malaria vector control involves measures to reduce the contact between the vector(s) and humans and to reduce the numbers of mosquitoes that reach the stage where they can transmit the parasite (infective stage). If done effectively, the transmission of the parasite is diminished and this reduces the number of people who develop malaria.

Current malaria vector control falls into two broad categories, shown in Table 1, and are mostly insecticide-based.

Table 1. Malaria vector control measures (adapted from WHO, 2006)

Method	Action	For individual and family protection	For community protection
Adult vector control	Reduction of human-mosquito contact	Insecticide-treated nets, repellents, protective clothing, screening of houses and other housing improvements	Insecticide-treated nets, zooprophylaxis
	Elimination of adult mosquitoes		Insecticide-treated nets, indoor residual spraying, space spraying, ultra low-volume sprays
Larval control (larval source Management)	Elimination of mosquito larvae	Peri-domestic sanitation	Larviciding of water surfaces, intermittent irrigation, sluicing, biological control
	Source reduction	Small-scale drainage	Environmental sanitation, water management, drainage

The major vector control methods currently implemented in malaria vector programs include methods targeting larvae and adult mosquitoes:

- **Larval source management (LSM):** This aims at reducing the number of vectors reaching the adult stage. LSM could be a good complimentary intervention particularly in situations of high mosquito population density with distinct and few breeding places, such as dry areas with limited and manageable sites (Table 2). LSM may involve:
  - Chemical insecticides (*e.g.* temephos), biological agents (*e.g.* bacteria such as *Bacillus thuringiensis israelensis - Bti*) or toxins that kill larvae and pupae.
  - Larvivorous fish such as *Gambusia affinis* and guppy (*Poecilia reticulata*).
  - Application of oil that forms a film on the water and thereby disrupts the ability of the larvae and pupae to breath.
  - Use of insect growth regulators that prevent larvae from reaching adulthood.

- Manipulating or physically eliminating larval habitats to prevent the breeding of mosquito. Where the changes are permanent (*e.g.* draining, filling in pools and ditches), it is called environmental modification.

- **Indoor Residual Spraying (IRS):** This targets the adult vector. It involves spraying the inner walls of houses with WHO approved insecticides with residual properties. Once applied, these insecticides dry up leaving a small film of crystals on the wall. The vector picks up the insecticide when it rests either before or after a blood meal and dies from the exposure, if susceptible to the insecticide. Some of the insecticides used for IRS are also able to repel the mosquitoes and this reduces the number of vectors entering into the sprayed rooms.
- **Insecticide Treated Nets (ITN):** This targets the adult vector. The net provides an effective barrier between the person who is sleeping under it and the mosquito vector. This reduces the opportunity for biting and infection. The impregnated insecticide also acts to kill and repel any susceptible vector that rests on the net. Currently, long lasting insecticidal nets (**LLINs**) are used which have a shelf life of about 2-3 years of use. It is normal for programs to target 80% and above of the population at risk in the area of operation, as this is has been shown to provide a community effect.

The effectiveness of each intervention depends on a number of variables that includes bio-ecological traits of the mosquito vectors, habitat characteristics of the area and socio-economic/cultural aspects of the human population. Table 2 provides some of the critical requirements for successful use of the three major interventions.

Table 2. Requirements for successful implementation of major vector control interventions (adapted from WHO, 2006)

Intervention	Requirement
Indoor Residual Spraying	• Vectors predominantly rest indoors (endophilic species) • Houses with walls and ceilings • Targeted population is not nomadic (permanent homesteads) • Effective community mobilization to maximize willingness of target population to accept spraying and comply with safety • Ability of the national program to organize the delivery of timely and correct application to all the houses in the targeted areas, including information on number and location of houses to be sprayed
Insecticide Treated Nets	• Most malaria infections are acquired indoors (endophagic species) • At least some of the vector biting occurs at hours when people are in bed • Effective community mobilization to maximize willingness of people to correctly use nets • An adequate system to deliver treated nets, including information on number and location of houses and sleeping units requiring nets • Ability to organize a net treatment program free of charge or to switch to use of long-lasting insecticidal nets
Larval Source Management	• Breeding in semi-permanent sites • Ability to locate and map out a very large proportion of the breeding sites within mosquito flight range of the community which it is required to protect • Proper selection of anti-larval measures • Community participation for breeding site reduction and/or elimination

1.2 Community education

Vector control measures should have a strong component of social participation that should normally aim at motivating personal and family protection and would include health education and community mobilization.

Measures aimed at reducing human-vector contact often imply a change in human habits. Educational programs focusing on the correct use of nets and other individual protective measures, sanitation, and on the need for correct therapeutics are usually undertaken with control programs.

1.3 Basic principles of planning malaria vector control and the role of entomology

The burden of malaria remains high in many places in spite of the efficacy of current control methods. Although there has been a significant increase in funding for malaria control in most endemic countries (from both external and internal country sources), resources generally still remain limited to national programs.

Malaria control strategies should be based on entomological and epidemiological studies which provide good information on the determinants of the local burden of disease. Most endemic countries, however, still face significant challenges to plan and implement vector control measures effectively. Infrastructure, technical skills and competencies remain inadequate. Furthermore, vectors in many places are developing resistance to insecticides.

Malaria entomology involves the study of the biological, behavioral and ecological factors that enable mosquito vectors to transmit malaria parasites from one person to another. It enables systematic investigation on why control measures that are being implemented may or may not be working. Entomology is therefore critical to planning and improving the disease control strategy.

Some questions that entomological studies will answer include:

- Identifying which *Anopheles* mosquitoes are present in the local area, and which of these species are responsible for transmitting malaria in the local area.
- The behavior (*e.g.* biting, resting habits) and the breeding habitats of local vector species: for example, whether the vectors feed on other animals apart from humans and what proportion feed outdoors compared with indoors.
- Whether or not the interventions that are being implemented are affecting the vectors and their ability to transmit malaria. Indicators measured will include changes in vector population density, rates of infection, susceptibility/resistance levels of vectors to the insecticides being used, and residual action of insecticides on treated surfaces and in impregnated nets.

Vector control programs are best planned on the basis of the outcomes of such entomological studies. Finally, implementation of vector control must place appropriate emphasis on cost-effectiveness and sustainability. Efforts must be made to progressively strengthen local capacities for planning, implementation, monitoring and evaluation.

Types of mosquito surveys

There are four main types of mosquito surveys:

- **Preliminary surveys:** These are original, basic and short-term. They are used to gather baseline data usually for the purpose of planning a vector control intervention. Emphasis on such surveys includes vector species identification, density changes, resting and feeding behavior, larval habitats, longevity, infection rates and insecticide susceptibility.
- **Regular or trend observations:** These are routine or long-term observations (longitudinal or operational surveys of monitoring). They are carried out regularly (*e.g.* weekly, monthly) in order to evaluate the impact of control measures.
- **Spot checks:** These are carried out in randomly chosen localities other than the fixed monitoring stations to provide supplementary information from areas otherwise not represented in routine monitoring.
- **Foci investigations:** These are carried out in areas of new or persistent malaria transmission to investigate reasons for disease transmission, or why implemented interventions are ineffective in reducing disease burden.

Unit 2
Biology of Malaria Vectors

Learning Objectives

Knowledge on the biology and behavior of *Anopheles* mosquitoes is important to understanding how malaria is transmitted and help in designing appropriate control strategies. This unit is aimed at providing basic knowledge on:

- **The disease and the parasite.**
- **The life cycle of the *Anopheles* mosquito.**
- **The larval habitats and conditions affecting the number of emerging adults.**

2.1 Malaria

Malaria is a major public health disease in most tropical countries. It is caused by parasites of the genus *Plasmodium* that are transmitted from one person to another through the bite of an infective female *Anopheles* mosquito. The male *Anopheles* feeds only on nectar and plant juices and thus does not transmit malaria.

There are five species of *Plasmodium* that infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi*. The later species is confined to Southeast Asia and infects mainly non-human primates.

There are about 480 species of *Anopheles* mosquitoes of which only about 80 are known to transmit malaria; 15 of these are considered major vectors of malaria. The mosquito picks up the *Plasmodium* parasite when it sucks the blood of an infected person. Once inside the mosquito, the parasite multiplies as it moves from the stomach of the mosquito to the salivary glands, from where it is passed on the next time the infected mosquito bites another person.

2.2 Life-cycle of the *Anopheles* mosquito

There are four stages in the life cycle of a mosquito: egg, larva, pupa and adult (Fig. 1). During its life-cycle the mosquito undergoes two changes (metamorphoses), from larva to pupa and from pupa to adult.

Egg stage

- The adult *Anopheles* female mates once and continues to lay eggs throughout its lifespan.
- Females must take a blood meal every 2-3 days. Blood is needed to develop eggs. Females will lay a batch of eggs before taking the next blood meal.

- Eggs are laid on water (rain pools, ponds, riversides, lakes, etc.) in batches of 50–200 eggs.
- The length of time the eggs take to hatch into larvae largely depends on temperature:
  - At about 30°C, eggs hatch into larvae in about 2-3 days.
  - In temperate zones (16°C), about 7-14 days.

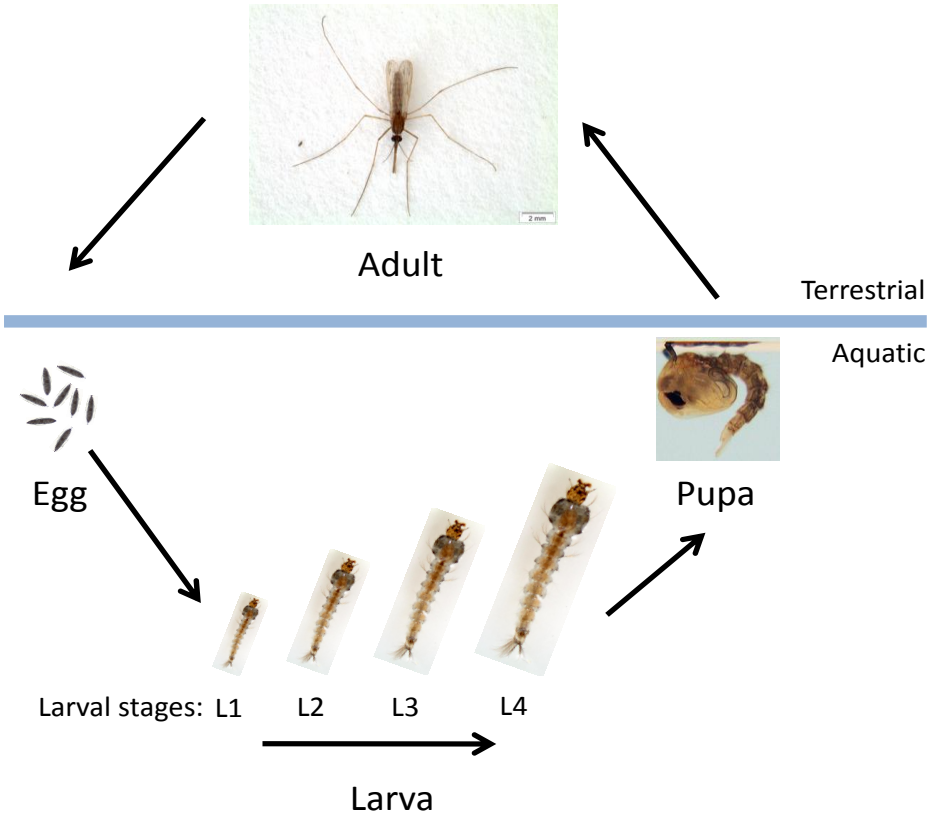


Figure 1. Stages of the *Anopheles* mosquito life-cycle

Larval stage

- The larva has a well-developed head with "mouth brushes" used for feeding (filter-feeders). The larva feeds on micro-organisms (*e.g.* algae, bacteria) and organic matter in the water where they breed.
- The *Anopheles* larva has no respiratory siphon. It lies parallel to surface of water in order to breath.
- There are four developmental stages of larva known as instars (denoted as L1 to L4, Fig. 1).

- The development from larva to pupa last about 5-10 days in normal tropical temperatures, depending on the species. Water temperature affects the time required for development, which is shorter in warmer waters.

Pupal stage

- The pupa is shaped like a comma and stays at the surface of the water.
- It has a pair of respiratory trumpets through which it breathes when at the surface.
- No feeding goes on during this stage but the pupa is motile and responds to stimuli.
- This is the resting or inactive stage during which there is a major transformation from living in water to emerging and living out of water.
- The pupa stage takes about 2-5 days.

Adult stage

- The adult usually emerges from the pupa at dusk.
- After emerging from the pupa, the adult mosquito rests for a short time in order to harden its body.
- Shortly after emergence the mosquitoes mate (Fig. 2). The males form large swarms, usually around dusk, and females fly into the swarms to mate.
- Both male and female mosquitoes feed on nectar for energy.
- After mating, the female mosquito searches for a blood meal for the development of her eggs. For some species one feed is enough to develop the eggs. In other species two feeds are required, at least for the development of the first batch of eggs.

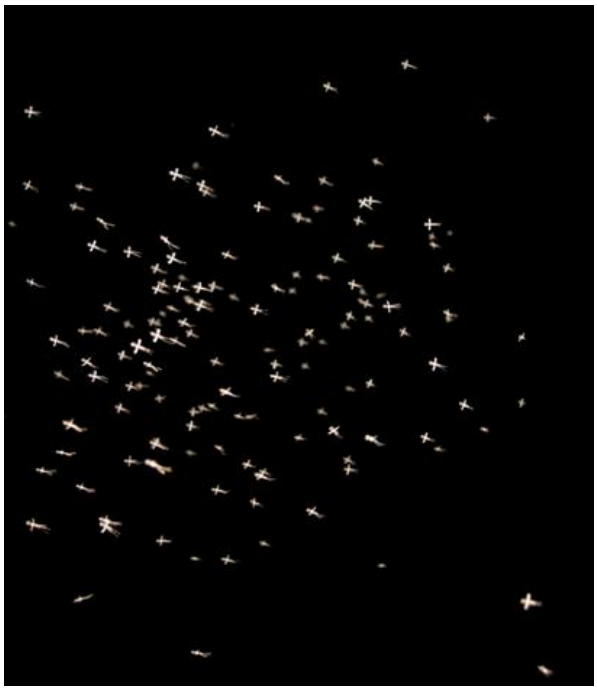


Figure 2. *Anopheles* male mosquitoes form swarms at dusk for mating

(photo: JD Charlwood)

- Duration from egg to adult *Anopheles* may vary between 7 days at 31°C and 20 days at 20°C.

2.3 Larval habitats and factors affecting adult production from aquatic habitats

The type of aquatic body suitable for mosquito larval development (larval habitat or breeding site), varies greatly between mosquito species and even within the same species. Some species prefer shaded water bodies while others prefer sunlit habitats. Some require unpolluted water

while others will breed in polluted water. Some species explore water bodies of a more permanent nature (*e.g.* drainages, water tanks, irrigation channels) and others occupy temporary puddles (Fig. 3).

Anopheles does not usually breed in swiftly moving streams or rivers, since larvae are not adapted to withstand wave action. But breeding sites can be as diverse as swamps, marshes, rice fields, temporary pools (puddles), ditches, drains, gulleys, rock-pools, tree holes, water storage containers and empty tins. However, some anopheline species show preference to specific conditions.

In Africa:

- *Anopheles gambiae* prefer small temporary water collections that are open to sunlight, such as puddles, hoof-prints or tire marks on dirt roads.
- *Anopheles funestus* prefer permanent or semi-permanent water bodies, usually with vegetation (*e.g.* edges of streams, swamps and marshes).

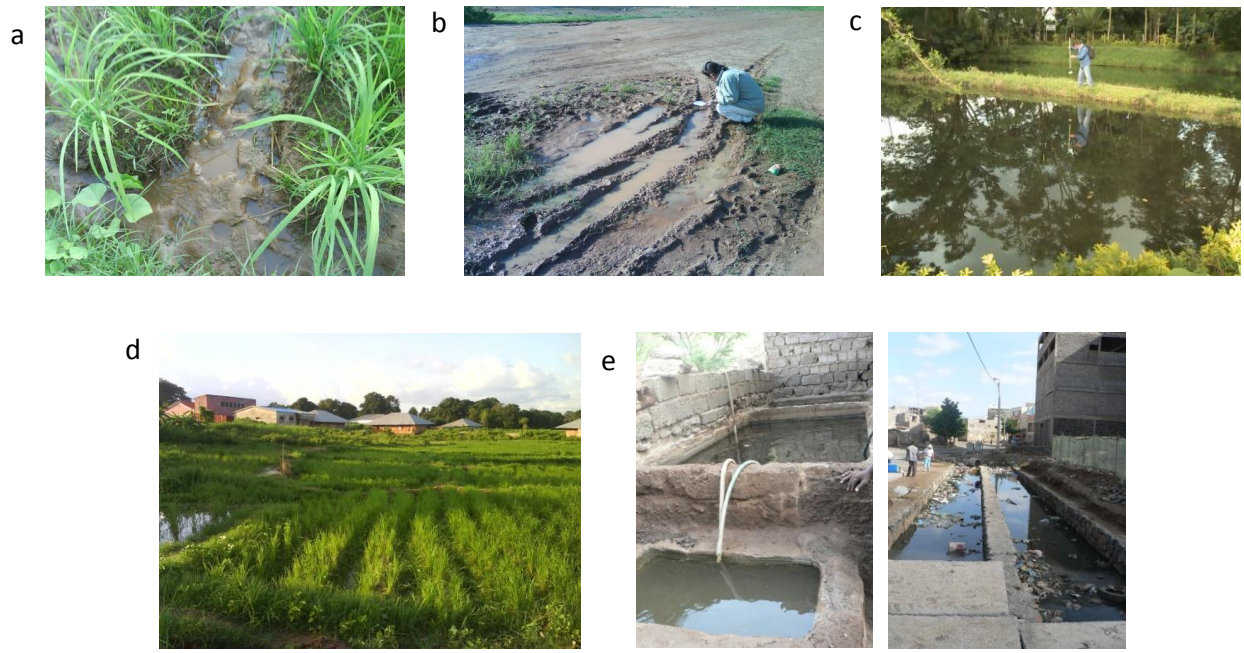
In the Americas:

- *Anopheles darlingi* larvae are mainly found in the shaded margins of streams and ponds with clear water and muddy bottoms, with emergent or floating vegetation.

In Asia:

- In urban areas, *Anopheles stephensi* breed in human-made habitats such as cisterns, wells, gutters and fountains presenting varying types of water, including polluted and brackish water.

Not much is known about the factors that affect the survival of larvae and the mechanisms that control the emergence of adults. Rainfall, temperature, humidity and time of year are however known to influence larval survival and emergence of adults.



a. Small puddle (temporary), b. Puddles on tire marks along a road (temporary), c. ponds (permanent), d. Rice field (semi-permanent), e. Water tanks and ditches (permanent)

Figure 3. Types of mosquito breeding sites

2.4 Characteristics of medical importance in adults

Longevity of adult *Anopheles* varies between species and depends on external factors such as temperature, humidity and presence of predators. The average lifespan of a female *Anopheles* is about 15 days, but lifespan of up to two months has been reported for some species.

Of great epidemiological importance are behaviors that concerning blood feeding and resting after a blood meal for egg development.

- Some mosquitoes bite predominantly indoors (endophagic) and others outdoors (exophagic).
- Some mosquitoes prefer to bite humans (anthropophilic), while others feed preferably on other animals (zoophilic).
- Mosquito species that tend to rest indoors during blood digestion and egg development are called endophilic while other species that rest outdoors are exophilic.
- Mosquito species may also differ in their biting activity during the night. Some species reach a peak of biting in the early hours of the night while other peak at dawn. Some mosquitoes begin biting at dusk, even before night falls. The daily biting pattern of a mosquito species is called its biting cycle.

Unit 3
Mosquito Anatomy and Identification

Learning objectives

By the end of this unit the participant should be able to:

- **Know how to identify adult *Anopheles* mosquitoes.**
- **Differentiate male and female mosquitoes.**
- **Distinguish the female *Anopheles* from other female mosquitoes.**
- **Distinguish between the egg and larva of *Anopheles* from other mosquitoes.**

Human malaria is transmitted exclusively by mosquitoes of the genus *Anopheles*. This genus belongs to a subfamily named Anophelinae (anophelines) within the family Culicidae. There is another subfamily called Culicinae (culicines) that includes two genera of great medical importance: *Aedes* (*e.g. Aedes aegypti*, vector of dengue and yellow fever) and *Culex* (*e.g. Culex quinquefasciatus*, vector of lymphatic filariasis). With the exception of the pupa, it is possible to easily distinguish anophelines from culicines at all stages of the mosquito life-cycle.

3.1 How to distinguish anopheline eggs from other culicines

- The *Anopheles* eggs have floats (Fig. 4) on the lateral sides and the eggs float separately in water.
- Culicine eggs do not have floats. Eggs of *Culex* species are laid in a raft that floats on the water surface. *Aedes* will lay individual eggs on solid surfaces and not on the surface of the water.

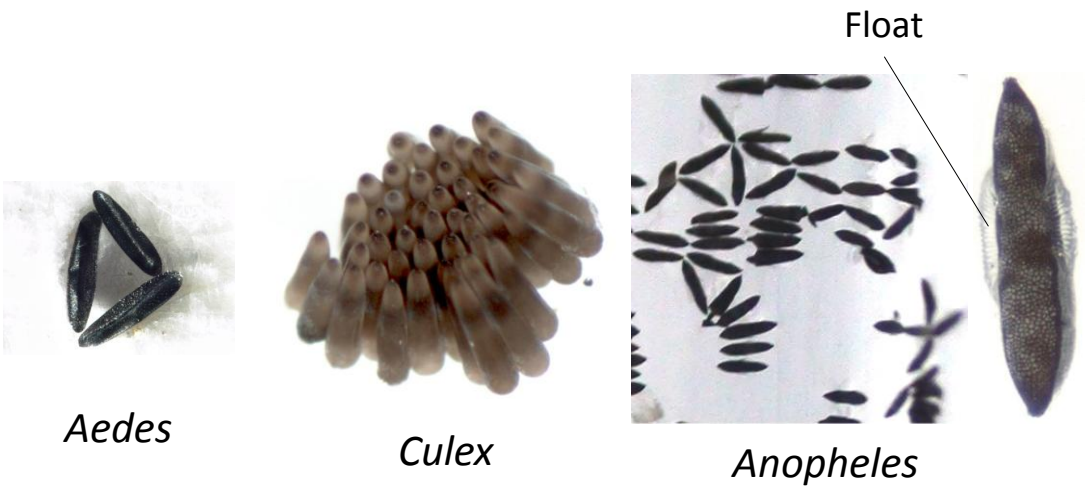


Figure 4. Examples of *Aedes*, *Culex* and *Anopheles* eggs

3.2 How to distinguish anopheline larvae from other culicines

The mosquito larva is divided in head, thorax and abdomen (Fig. 5). During its development, the larva passes through four instars (L1-L4) with an increase in size between instars (Fig. 1).

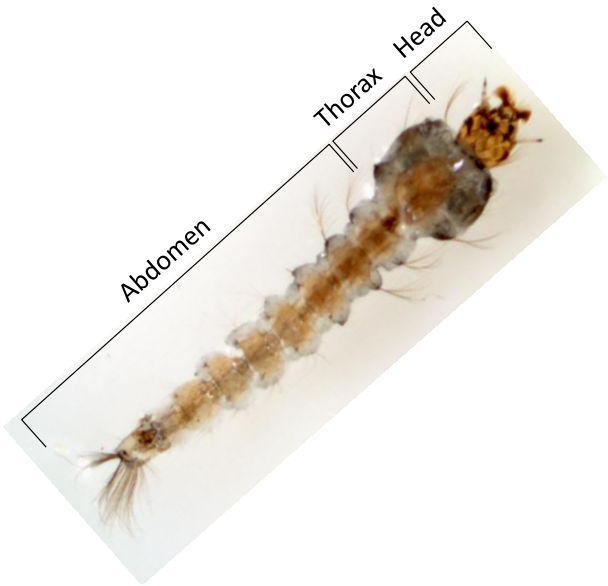


Figure 5. Anatomy of *Anopheles* larva

Two main features distinguish anopheline from culicine larvae (Fig. 6):

- Culicine larvae (*Culex* and *Aedes*) have siphon tubes for breathing and hang from the water surface.
- Anopheline larvae do not possess a siphon and they rest parallel to the water surface. Instead of a siphon they breathe through small opening called spiracles.

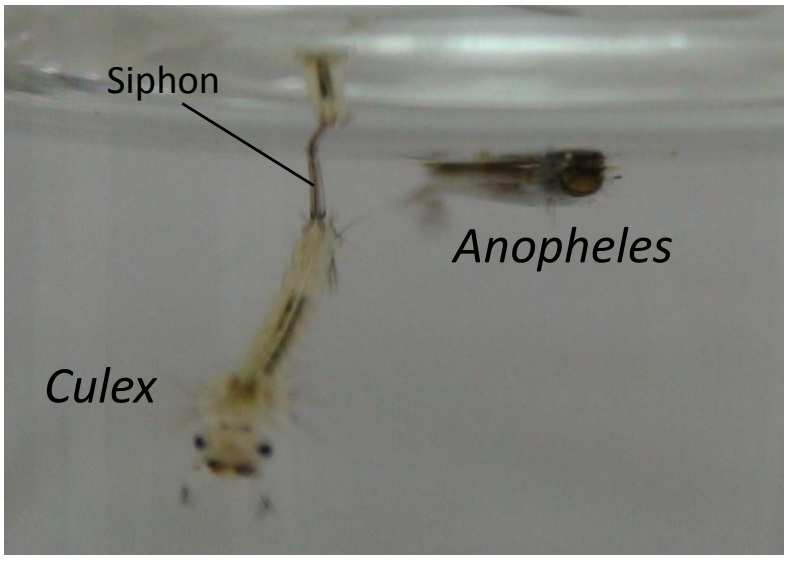


Figure 6. Differences between anopheline and culicine larvae

3.3 The pupa

The mosquito pupa is shaped in a comma (Fig. 7). They rest just at the surface of the water and swim briskly when disturbed. It is very difficult to distinguish between anopheline and culicine pupae, as the differences between them are more subtle.

Figure 7. A pupa of *Anopheles*

3.4 How to distinguish adult anopheline and culicine mosquitoes

The adult mosquito body is divided into head, thorax and abdomen (Fig. 8). The main components of the head include two large compound eyes, two antenna, two maxillary palps and the proboscis, which is adapted to pierce and suck. In the thorax, there are three pairs of legs (hind, mid and fore), one pair of wings and one pair of halters (modified vestigial wings). The abdomen is composed by 10 segments and the last two are modified to form the genitalia (male or female).

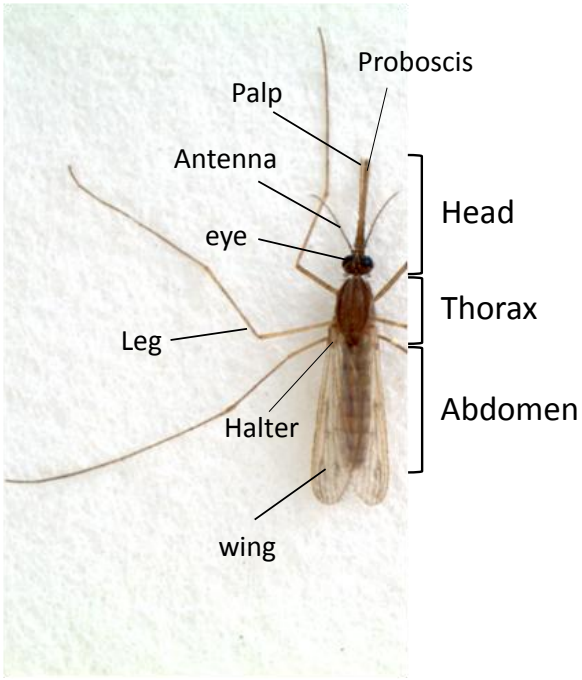


Figure 8. Anatomy of an adult mosquito

There are two main features that can be used to distinguish between adult anophelines and culicines: the maxillary palps (Fig. 9) and the resting position (Fig. 10).

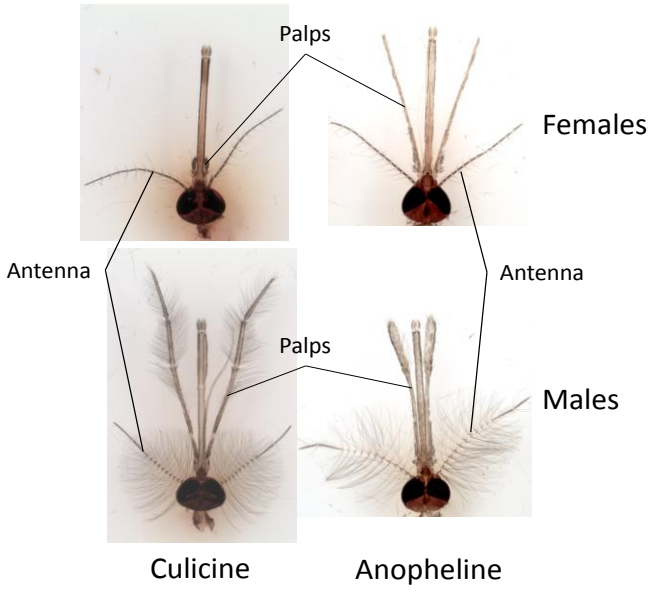


Figure 9. Differences in the head of male and female anopheline and culicine mosquitoes

- Female species of the genus *Anopheles* have maxillary palps that are as long as the proboscis. The culicine females have palps much shorter than the proboscis.
- The tip of the palps of the male *Anopheles* is club-shaped. That of culicines is not.
- Common to both anopheline and culicine mosquitoes is the sexual dimorphism of the antenna. Males have bushy (plumose) antenna while females have simple (pilose) antenna (Fig. 9).
- Adult *Anopheles* mosquitoes tend to rest at an angle of between 50^0 and 90^0 to the surface. Culicines tend to rest about parallel to the surface (Fig. 10).

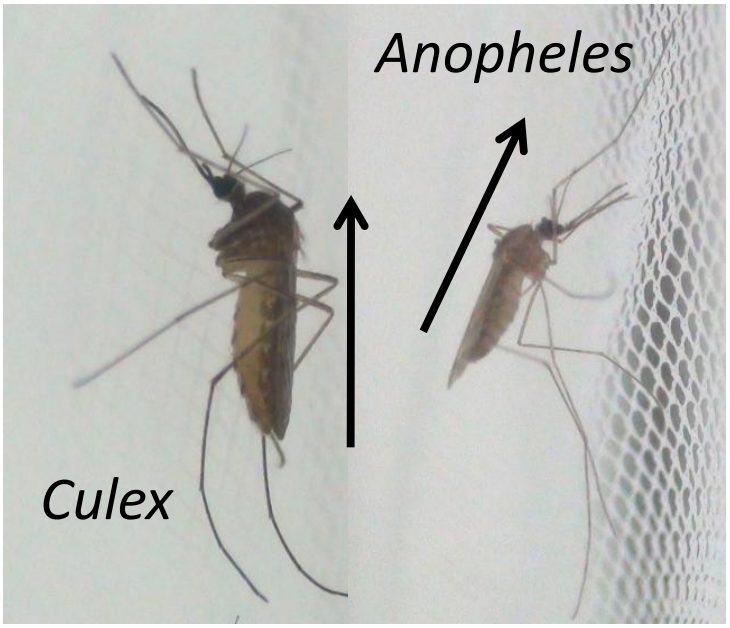


Figure 10. Resting position of culicine and anopheline adult mosquitoes

3.5 Methods for mosquito species identification

Apart from the above-mentioned distinctions between anophelines and culicines, it is also important to distinguish between different species of anophelines. Several methods can be applied in the identification of *Anopheles* species. These include:

- **Morphological methods** using taxonomic keys. Some of the main morphological characters used in mosquito identification are located in the palps, proboscis, legs, wings and thorax. Taxonomic keys are available for the identification of local malaria vector species and these keys may differ according to the geographical area and the vector species present.
- **Cytotaxonomy**. These methods use species-specific differences in the chromosomes. These techniques can only be applied to certain stages of the mosquito life-cycle or

sexes, when polytene or "giant" chromosomes are available for observation under a microscope.

- **Molecular methods.** These methods consist of the analysis of species-specific differences at the DNA level. Since DNA remains unaltered during the mosquito life-cycle, these methods can be applied in any life-stage (immature or adult) of the mosquito.

The cytotaxonomic and molecular methods of identification are normally applied to identify groups of species that do not display morphological differences between each other, such as those that compose sibling species complexes (see Unit 4). These methods will not be demonstrated in this course.

Unit 4
Diversity of Malaria Vectors

Learning objectives

Knowledge of the local malaria vectors is important for understanding transmission and for developing effective control strategies. This unit will help to understand:

- **That malaria transmission, in many cases, is sustained by several vector species that co-exist in the same area or region.**
- **Different species display different behaviors, which may affect malaria transmission and control.**

Anophelines comprise approximately 480 species, of which only about 80 are considered malaria vectors. With the exception of the Antarctica, there are malaria vectors in all continents of the world. More importantly, several vector species can occur in the same area and at the same time (sympatric species).

Due to differences in ecology and behavior, sympatric species may form complex vectorial systems. For example, when a vector species that explores semi-permanent breeding sites co-occurs in the same area with a species that prefers temporary puddles, they pose increased difficulties to larval-based vector control. Similarly, the sympatric occurrence of an endophagic species with an exophagic one creates additional challenges to the effectiveness of indoors-based vector control such as LLINs.

Correct identification of the target vectors is therefore critical to the successful implementation of any vector control strategy.

4.1 Sibling species complexes

Anophelines comprise several species that look morphologically the same, but have different genetic make-up. These species are called sibling or cryptic species and together they are referred to as a 'complex' or 'group'. In spite of being morphologically identical, sibling species are reproductively isolated. This results in the accumulation of genetic differences that often leads to differences in the bio-ecology and behavior. These differences may result in different medical importance of the sibling species of a complex and can also have important implications in vector control.

Depending on the geographic region, the *Anopheles* species composition varies, and hence the vectors responsible for the transmission of malaria also vary from one region to another. In the following sections, a brief description will be given of some of the major human malaria vectors in Africa, the Americas and Asia. The species descriptions were based on the works of Service

and Townson (2002), Manguin *et al.* (2008), Hay *et al.* (2010) and Sinka *et al.* (2010a,b). Students are encouraged to consult these works to obtain a more complete and detailed view of the malaria vector species diversity in the different geographic regions of the world.

4.2 Malaria vectors in the Americas

Among the major mosquito vectors responsible for malaria transmission in the Americas are the following species:

Anopheles albimanus

This species is an important malaria vector in Mexico, Central America and in the northwestern part of South America (Colombia, Ecuador, Peru and Venezuela). Typical larval habitats are open, sunlit, natural or human-made sites with clear fresh or brackish water, usually containing floating or emerging vegetation. This species bites both indoors and outdoors and is mainly exophilic. It has a tendency for zoophily but this greatly depends on geographic location and host availability.

Anopheles albitarsis complex

The *Anopheles albitarsis* complex comprises four species: *Anopheles albitarsis* A and B, *Anopheles marajoara* and *Anopheles deaneorum*. Larvae grow in large sunlit pools, ponds, rice fields and marshes with fresh and clear water and filamentous algae. Adults are exophilic and bite readily both humans and domestic animals, indoors and outdoors. This complex can be found throughout most of the north, east and central parts of South America.

Anopheles darlingi

While this species is widespread, it is the major malaria vector of the Amazonian region. Its distribution spans from the north of the continent (Colombia, French Guiana, Guyana, Suriname, Venezuela and northern Peru) to the east of Brazil and southwards to Paraguay and northern Argentina. It is a riverine mosquito adapted to rural and forested areas. Characteristic breeding sites are the shaded edges of slow flowing streams with clear water and submersed vegetation, but it can also be found in freshwater ponds, marshes, lagoons and rice fields. *Anopheles darlingi* tend to rest outdoors and its degree of endophagy as well as anthropophily is quite variable. In some occasions this variation has been associated with behavioral changes in the human population.

Anopheles nuneztovari complex

This complex includes either two or possibly three sibling species (A and B/C) identified by chromosomal differences, but the taxonomic status of this complex is yet to be fully clarified. The complex is distributed along most of the northern and central parts of South America, being absent in the east and west coastal areas of the continent. Larval habitats are usually sunlit turbid water pools, vehicle tracks, hoof prints and small ponds of temporary or semi-permanent

nature. Adults are mainly exophilic, exophagic and zoophilic, but can bite humans outdoors. There is a difference in the biting cycle between the sibling species, with species A being an early biter (biting peak: 6-8pm) and species B/C a late biter (biting peak: 10pm-2am).

Anopheles pseudopunctipennis complex

This complex comprises at least two sibling species and has a widespread distribution from the southern USA, across Central America, the eastern part of the South American continent and down to the north of Argentina. It can survive at high altitudes (up to 3000m). Larvae are found mostly in sunlit edges of shallow freshwater streams and river pools where abundant filamentous algae provide protection. This species may be a major vector during the dry season, when river levels are low and small pools form. Adults display a marked opportunistic biting behavior, feeding on both humans and animals, indoors and outdoors. They are considered mainly exophilic but several studies suggest that a proportion of mosquitoes of this species will rest indoors after feeding.

4.3 Malaria vectors in Africa

In Africa, the major malaria vectors are members of the *Anopheles gambiae* complex and the *Anopheles funestus* group. Given the great importance of malaria in the African continent, these are probably the most well studied mosquito species in the world.

Anopheles gambiae complex

This complex comprises 7 sibling species that can be grouped into freshwater species: *Anopheles gambiae* sensu stricto, *Anopheles arabiensis*, *Anopheles bwambae* and *Anopheles quadriannulatus* A and B; and brackish water species: *Anopheles melas* and *Anopheles merus*.

- ***Anopheles gambiae* s.s. and *Anopheles arabiensis***

  Anopheles gambiae s.s. and *Anopheles arabiensis* are the main malaria vectors of the complex and have the widest geographic distribution. *Anopheles gambiae* s.s. predominates in forest and humid savannah zones whereas *An. arabiensis* is more successful in arid environments. Both species explore temporary breeding sites, usually small, shallow, sunlit and without vegetation. Both species often occupy the same larval habitat. *Anopheles gambiae* s.s. feeds mostly on humans (anthropophilic). *An. arabiensis* is generally more zoophilic. However, the species shows high variability in host preference and biting behavior across Africa. With few exceptions, *Anopheles gambiae* s.s. is generally endophagic and endophilic. *Anopheles arabiensis* displays a greater variation concerning these behaviors.

- ***Anopheles quadriannulatus* A and B**

  Anopheles quadriannulatus A is strictly zoophilic and thus it is the only member of the *An. gambiae* complex that does not transmit malaria. The egg-laying sites are similar to those of the other freshwater species of the complex. In 1998, a new species was described from

samples collected in Ethiopia and provisionally named as *Anopheles quadriannulatus* species B. Very little is known about its biology.

- ***Anopheles bwambae***

  This species breeds in water derived from hot springs at temperatures of 33-36°C and with slightly higher pH than freshwater sites explored by larvae of *An. gambiae s.s.* The distribution is confined to the Semliki forest of Uganda. High densities prevail perennially in the forest, where it bites humans mainly outdoors. Although the species is able to transmit malaria, it is not a very important vector due to its local distribution.

- ***Anopheles melas* and *Anopheles merus***

  These are the two brackish water-adapted species of the complex. Both occupy coastal habitats with mangrove belts (*e.g.* in estuaries, lagoons and swamps). However, they differ in the geographic distribution. *Anopheles melas* occurs in the west African coast whereas *An. merus* is restricted to the coast of east Africa. Both species are considered secondary vectors of malaria.

Anopheles funestus group

The *Anopheles funestus* group comprises nine sibling or closely related species. Of these, only the nominal species, *Anopheles funestus* s.s. is a malaria vector throughout Africa. None of the following group members are vectors of malaria: *Anopheles rivulorum* (West and East Africa), *Anopheles leesoni* (West and East Africa), *Anopheles confusus* (East Africa), *Anopheles parensis* (East Africa), *Anopheles vaneedeni* (North of South Africa), *Anopheles fuscivenosus* (Zimbabwe), *Anopheles aruni* (Zanzibar) and *Anopheles brucei* (Nigeria). These species are mainly zoophilic.

- ***Anopheles funestus s.s.***

  It is considered the second most important vector of malaria in Africa, after *An. gambiae* s.s. Like the later species, it has a widespread distribution throughout the African continent south of the Sahara desert. *Anopheles funestus* s.s. typically breeds in relatively large permanent and semi-permanent water bodies with vegetation (*e.g.* swamps, ponds, lake shores). It is a highly anthropophilic species that bites mostly indoors (endophagic).

4.4 Malaria vectors in Asia

Some examples of main malaria vectors of the Southeast Asian region are:

Anopheles culicifacies complex

The *An. culicifacies* complex has a widespread distribution throughout the Asian continent, ranging from Ethiopia and the southern coast of the Arabian Peninsula eastwards through the Indian subcontinent and to southern China, Vietnam, Laos, Cambodia, Thailand, and Myanmar. Five chromosomally recognized species (A, B, C, D and E) have been described in this complex. Of these, species E is considered the most important malaria vector of the complex,

particularly in India. Species B is a non-vector. Larvae occupy a variety of breeding sites: clean and polluted water, sunlit or shaded. Species E is highly endophilic and anthropophilic, whereas the other species are more zoophilic, especially species B. Biting occurs both indoors and outdoors.

Anopheles dirus complex

This complex includes seven sibling species: Anopheles dirus, Anopheles cracens, Anopheles scanloni, Anopheles baimaii, Anopheles elegans, Anopheles nemophilous and Anopheles takasagoensis. With the exception of An. elegans (which is found in hilly forests of southwest India), An. baimaii (from northwestern India to southern Myanmar and western Thailand) and An. takasagoensis (Taiwan) the remaining species of this complex are distributed across the Indochina and Malay peninsulas. Larvae usually breed in small temporary and shaded puddles and hoof prints, in or at the edges of forests. The complex includes both major vectors of malaria in areas of rain forest, cultivated forest and forest fringes, and species of little or no importance as malaria vectors. Anopheles dirus and An. baimaii are main vectors of forest malaria, being mainly exophagic and anthropophilic. They tend to rest outdoors after feeding. Anopheles nemophilous and An. takasagoensis are zoophilic species and are thus considered to be non-vectors.

Anopheles maculatus group

The An. maculatus group includes 8 sibling species, 6 of which form two subgroups: the maculatus subgroup (Anopheles dispar, Anopheles greeni, Anopheles dravidicus, and Anopheles maculatus); the sawadwongporni subgroup (Anopheles notanandai and Anopheles sawadwongporni). Two additional species are not assigned to a subgroup: Anopheles pseudowillmori and Anopheles willmori. The members of the An. maculatus group are distributed throughout Asia, from India to Indonesia and the Philippines. Precise definition of the relative role of each species in malaria transmission has been difficult due to misidentification problems.

- ***Anopheles maculatus***

  The nominal species of the group has the most widespread distribution, ranging from Western Afghanistan and Pakistan eastwards to southern China and Taiwan and southwards into the Indochina and Malay peninsulas and Indonesian islands (Sumatra and Java). It is considered a major malaria vector in eastern India, southern Thailand, Malaysia, and Java. This species is mainly found in or near hilly and mountainous areas, where it uses a variety of larval habitats, including seepage waters, ditches, rice fields, ponds, stream margins, swamps and lakes. Adults bite animals and humans, both indoors and outdoors, and resting after feeding occurs outdoors.

Anopheles minimus complex

This complex is formed by at least three sibling species: *Anopheles minimus* species A, *Anopheles harrisoni* (species C) and *Anopheles minimus* species E. The distribution of the complex extends from the northwest of India eastwards to Bangladesh, Vietnam, Laos, Cambodia, Thailand, Myanmar and southern China, and southwards to Malaysia and Indonesian islands. *An. minimus* and *An. harrisoni* are responsible for malaria transmission in hilly regions at altitudes between 200-1000m. They occur in forested areas where larvae breed in slow-flowing clear water streams with grassy margins, but can also exploit water tanks, rice fields and borrow pits. *Anopheles minimus* is considered to be anthropophilic, endophagic and endophilic, but displays high variation in trophic behavior. Compared to it sibling species, *An. harrisoni* appears to be more zoophilic and exophagic.

Unit 5
Mosquito Collection (Larvae)

Learning objective

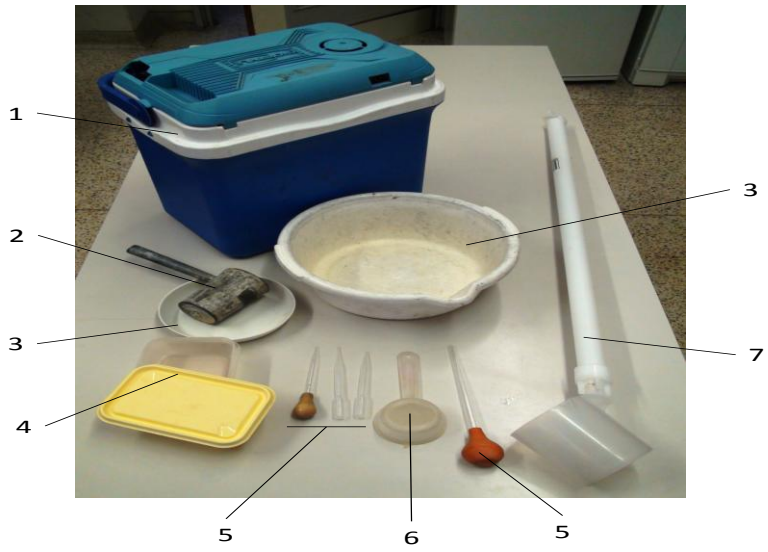
This unit will provide basic understanding on how to:

- **Sample larvae and pupae of mosquito vectors from natural habitats.**

Various mosquito vectors display different larval habitat preferences. Breeding sites can be very diverse, including ponds, lakes, swamps, marshes, rice fields, small rain pools, hoof-prints, car tires, tree holes, plant axils and edge of streams. It is important to know the breeding preferences of the local vectors of malaria in order to implement effective control measures. Mosquito larval collection is a critical activity in vector surveillance. The information obtained from larval collections includes:

- Determination of the vector species present in the study area.
- Identification of preferred active breeding sites for each species.
- Determination of the geographical distribution of vectors.
- Evaluation of anti-larval measures on larval density.
- Collect samples for rearing adults in the insectary.

Sampling equipment required for larval collections depend on the type of method used. The most common equipment and materials include (Fig. 11): dippers, nets (netting for larger concentration of larvae), quadrants, trays, strainers, and ladles for small water collections, pipettes, covered containers (for holding samples) and recording materials (water proof markers, tapes, recording forms, see section 5.2). A GPS, water thermometer and pH meter are also useful to locate and characterize the breeding sites. Collectors should wear rubber boots and use protective gloves when sampling.



1. cool box, 2. ladle, 3. trays, 4. covered container, 5. pipettes, 6. strainer, 7. dipper.

Figure 11. Main materials for larval collections

5.1 Sampling methods

Several larval sampling methods are available. The use of each sampling method depends on the nature and type of breeding site and is described in the subsequent paragraphs.

The larval collector must approach the breeding site carefully, as any disturbance will cause the larvae and pupa to swim downwards and become inaccessible. It is important that the collector does not cast a shadow on the water. If the larvae and pupae move, it may be necessary to stand quietly until they swim back up.

Dipping

- This method is normally used to sample from relatively large water bodies such as swamps, ditches, streams and rice fields (Fig. 12).
- The dipper should be lowered gently at an angle of about 45^0 to minimize disruption and either skim the top of the water or gentle lower it to cause the water and nearby larvae to flow into the dipper. Care should be taken not to spill water when raising the dipper from the water.
- Larvae should be collected from the dipper using a pipette and transferred to a well-labeled bottle or vial.
- When the breeding site has emerging vegetation, the collector should disturb the water and cause the larvae to swim downwards, then remove some vegetation to create a clear spot for sampling, and wait for a few minutes before resuming sampling as previously described.

- The number of dips in each breeding place should be annotated for calculation of larval density. Note also the amount of time spent on collection.

Figure 12. Larval collection by dipping

Netting

- This method consists of using a fine mesh net mounted on a handle, with a plastic bottle or tube tied to one end. It is normally used to collect larvae and pupae in larger water bodies such as ponds and small lakes.
- The net should be held at about 45° angle to the water surface and dragged across the surface. The larvae and pupa are collected in the plastic bottle at the end.

Pipetting:

- This method is used for collecting larvae in small breeding sites such as small puddles, hoof-prints, containers, plant axils and tree holes (Fig. 13).

Figure 13. Larval collection by pipetting

5.2 Records of collection

Where feasible, use a GPS or other manual means (*e.g.* drawing) to locate and number the breeding sites sampled. The characteristics of a breeding site should be annotated, namely:

- Geographic location (GPS coordinates, name of the locality).
- Type of breeding site (permanent, semi-permanent, temporary).
- Origin of the water (*e.g.* rain, river, lagoon, man-made).
- Nature of the water collection (*e.g.* puddle, rice field, ditch).
- Exposure to sunlight (shaded, sunlit).
- Presence of vegetation (emergent, submerged, floating).
- Characteristics of the water (*e.g.* clear, turbid, polluted, dark, temperature, pH)

Data recorded should also include the number of dips made, the time spent sampling and the date. All vials/bottles containing larvae from a breeding site should be labeled with the number of the breeding site that was annotated in the notebook. An example of a data recording form for larval collections can be found in Annex II.

5.3 Transportation of live larvae

All specimens collected from a particular breeding place should be kept in one bottle or vial and labeled in pencil. The label is dropped inside the vial. The label should include critical identifiers such as date, location, initials of the collector, sample number. This should be reflected on the data form. In addition, the side of the vial can also be labeled with a permanent ink marker.

- To prevent undue shaking or exposure to extreme heat, vials/bottles should be transported in an appropriate container such as a thermal box with cold packs.
- When transporting larvae for long distances, do not cover the container. If covered then open the container at intervals (*e.g.* every 2 hours). Ensure that there is about 1-2cm space in the bottle or vial to allow air for larvae and pupae to breathe.
- In some occasions, carry water from the breeding site in another container, especially if larvae are to be used for susceptibly tests (thus needing to be reared to adults).

5.4 Preserving samples

In the laboratory, larvae are identified, counted and preserved for subsequent analysis. Larvae may be killed by heat (by placing them in hot water 50°C-70°C) or by drowning in absolute ethanol. Larvae should then be preserved in ethanol 70-80% or ethanol 80% + glycerin 2%. If hot water is used, then pour the water out of the vial, being careful that the now-dead larvae remain in the vial. Then add the ethanol into the vial and close it.

5.5 Estimation of larval parameters

Larval surveys provide several pieces of information on bio-ecological aspects of mosquito species. They are also used to assess the impact of vector control measures, by comparing larval densities and breeding site occupation before and after the implementation of the intervention.

The estimation of larval densities is complex, as it requires standardization of the sampling effort. For example, this may imply performing the same number of dips at each breeding site and using dippers of the same size. This can be difficult in the field, since breeding sites may vary greatly in size and shape. To overcome these limitations, more elaborate sampling strategies and devices may be required for estimating larval densities. When the correct sampling conditions are met, a simple index for larval density is the Mosquito Breeding Index (BI)[3]:

$$BI = TLP \div ND \times BP$$

Where:

TLP = total number of larvae and pupae collected
ND = total number of dips performed
BP = Number of breeding sites sampled

[3] Belkin J.N. (1954). Simple larval and adult mosquito indexes for routine mosquito control operations. *Mosquito News* 14:127-131.

Other parameters are available to estimate the occupation of breeding sites by mosquitoes. Three examples are given below[4]:

General breeding index (GBI)

This index gives a measure of the proportion of water bodies that mosquitoes use for breeding at a given locality. It is calculated by dividing the number of breeding sites with immature culicids (larvae and pupae) by the total number of breeding sites surveyed.

Absolute breeding index (ABI)

This is the relative proportion of breeding sites occupied by a given vector species in a locality. Calculation of this index requires that larvae are identified to species level. It is obtained by dividing the number of breeding sites positive for that species by the total number of breeding sites surveyed.

Relative breeding index (RBI)

This parameter indicates the abundance of the breeding sites of a given species in relation to the number of water bodies where mosquitoes are found in a locality. It is calculated by dividing the number of breeding sites positive for that species by the total number of breeding sites positive for mosquitoes.

[4] Ribeiro H. *et al.* (1980). *Os mosquitos de cabo verde (Diptera: Culicidae), sistemática, distribuição, bioecologia* e *importância médica.* Junta de Investigações Científicas do Ultramar, Lisboa. 141p.

Unit 6
Mosquito Collection (Adult)

Learning Objective

This unit will provide basic information on:

- **Methods used for sampling adult mosquitoes.**

Mosquito populations in any locality are made up of different individual species, exhibiting different behaviors and physiological states (*e.g.* fully-fed, gravid and newly-emerged). Different behaviors may also be displayed according to the physiological state of the mosquito vector. This includes host seeking, resting for egg maturation and exiting from houses in search of a site to lay eggs (oviposition site). Various sampling methods have been devised to take into account these differences within the populations.

6.1 Adult mosquito collection methods

Before heading out to the field, there is a need to ensure that all essential equipment for adult mosquito collection is assembled. There will be a class demonstration on how to prepare the materials used in each collection method and how to handle them in a correct manner. Students will have opportunity to practice these prior to the field work classes.

Human landing catches (HLC)

This method standardizes a procedure for evaluating the interactions between the vector and the human host. The number of vectors biting humans is a major parameter in estimating the level of malaria transmission, as it helps to answer the following:

- Which anopheline mosquitoes bite humans.
- Which species are malaria vectors.
- How often a person is bitten by a vector.
- The biting pattern during the night.
- Whether vectors bite indoors or outdoors.

Essential materials (Fig. 14): Mouth/mechanical aspirators, un-waxed paper cups with net covering the opening, rubber bands, torchlights (flashlights) and spare batteries, test-tubes (110mmx10mm or 60mm x 10mm) and rubber stoppers (as alternative to aspirators and cups), cotton wool, mosquito netting, 10% sugar solution, pencil or permanent ink marker, adhesive tape.

1. mouth aspirator, 2. mechanical aspirator, 3. flashlight, 4. spare batteries, 5. adhesive tape, 6. rubber bands, 7. paper-cups with netting, 8. cotton wool.

Figure 14. Main materials for hand collection of mosquitoes

Landing catches involve a team of two or more people sitting either indoors or outdoors and collecting mosquitoes off themselves, as the mosquitoes attempt to bite (Fig. 15). Alternatively a pair of collectors will work together in which one collector will lie down with his legs exposed to the knee, while the other sucks the mosquitoes that land on his partner with an aspirator.

Figure 15. Human landing catch

These collections are usually carried out during the evening/night, to follow the biting cycle of *Anopheles* mosquitoes. Whenever possible, teams of collectors are placed outdoors and inside houses (indoors). Collections are made either throughout the night or part of the night as the study objective may require. Indoor collections are done from 6pm to 6am, while the outdoor collections may be done from 6pm to 10pm, with the assumption that people turn in to sleep in their rooms by 10pm and thus are not at risk to outdoor biting after 10pm. However, in communities where people tend to normally sleep outdoors, either because of hot weather or other reasons, then it may be reasonable to conduct the outdoor biting collection from 6pm to 6am as well.

Collectors expose the legs to the knee to serve as bait and sit as quietly as possible. Once the collector feels the mosquito landing, the flashlight is turned on to see the mosquito, which is then collected with the aspirator and placed inside the net-covered paper cup. It is not necessary to allow the mosquitoes to bite/feed, as they are collected as soon as they land on the collector. Hence the measurement relates more to landing rather than feeding.

A different paper cup should be used for each hour of collection and labeled accordingly. This will allow counting of how many mosquitoes were collected at which hour of the night. The collected mosquitoes will then be sorted according to species the next morning. The samples are segregated by species, by house, by day and by hour of collection.

The limitations of landing collections include variation in the attractiveness of human hosts/baits to mosquitoes and ethical considerations regarding accidental infection with malaria. To overcome the first point, collectors should switch sites every hour or rotate in batches. The collectors are usually on antimalarial prophylaxis to prevent malaria cases. Also, there should be

prompt access to treatment with effective malaria medicines in the event that any of the collectors gets malaria.

Pyrethrum Spray Sheet Collection (PSC)

Pyrethrum spray catches are used to estimate the numbers of mosquitoes resting inside rooms where people slept the previous night. These collections are usually done during the morning. Samples obtained from PSC enable:

- Determination of the physiological condition of the abdomen of mosquitoes. The stage of their abdomen gives an indication of the resting and feeding behavior. The vectors could be unfed, fully blood fed, half-gravid or gravid, depending on how long they have stayed in the room.
- Determination of the seasonal room density of mosquito vectors.
- An indirect measurement of man-biting density, where the vector is highly endophilic (indoor resting).

Essential materials: Torch/flashlight and spare batteries, Petri-dishes, white cotton sheets (2mx1m, 2mx2m, 2mx3m), hand sprayers (double-action type), insecticide (pyrethrum 0.2-0.3% in kerosene), forceps, cotton wool, filter papers, labels and container for carrying samples.

Prior to spraying, all animals should be removed, all food covered and small furniture removed from the room the collection will take place. White sheets are then laid to completely cover the floor and all flat surfaces (ensure that the sheet is spread under tables as well). All windows and doors should be closed.

The operator then carefully sprays in a clockwise direction towards the ceiling until the room is filled with a fine mist. The operator exits the room rapidly, closes the door and waits for about 10 minutes.

Beginning from the entrance, the corners of the sheet are lifted and the sheet is taken outside. All knockdown mosquitoes are collected in the daylight with forceps and placed in a labeled Petri-dish, on top of a layer of damp cotton wool and filter paper (Fig. 16).

Mosquitoes collected in each house are stored in separate Petri-dishes appropriately labeled (e.g. collection date and hour, village, household number/name of head of household).

Figure 16. Pyrethrum spray sheet collection

Outdoor resting collection (ORC)

The method is used to sample mosquitoes resting outdoors in their natural resting places (exophilic) (Fig. 17). Because some vectors feed indoors and rest outdoors and others feed outdoors and rest outdoors, normally the source of blood meals of outdoor resting mosquitoes will indicate host preference and feeding behavior. Data from outdoor collections are important in evaluating the impact of anti-vector measures and provide information about:

- The species that habitually rest outdoors.
- The proportion of mosquitoes resting outdoors.
- Seasonal changes in outdoor resting habitats.
- Changes in the relative numbers of mosquitoes resting outdoors following the application of insecticides inside houses.

Essential materials: Torch/flashlight and spare batteries, battery-powered mechanical or backpack aspirators, un-waxed cups with net covers to store collected samples, pencil or permanent ink marker for labeling and a container to transport samples.

a  b 

a. from the vegetation with a backpack aspirator b. in a corral with mechanical aspirators (photo: C.A. Sousa)

Figure 17. Outdoor resting collections

Collectors should look for resting mosquitoes in suitable outdoor resting places. These are usually shaded and humid sites, such as the surrounding vegetation, tree holes, crab holes, or outer walls of households near the roof, as well as in domestic animal shelters (corrals, stables). Collections are usually performed during the day, typically in the morning but sometimes in the early evening, depending on the vector species.

Hand collection (aspiration) of indoors resting mosquitoes (IRC)

Hand collections are usually performed using mouth or battery-powered mechanical aspirators (Fig. 18). The mosquitoes are sought and collected from bedrooms and other walls of the house and furniture with the aid of torch/flashlight. These provide the following important information:

- The species and proportion of mosquitoes that rest indoors.
- Indoors resting density, usually expressed as the number of resting mosquitoes per collector, per hour.
- Seasonal changes in indoor resting density.
- Changes in the relative numbers of mosquitoes resting indoors following the application of insecticides inside houses.

Essential materials: Torchlight/flashlight and batteries, paper cups, mechanical or mouth aspirators/test tubes, cotton wool, labels, 10% sugar solution.

Collections are usually done first thing in the morning when the occupants leave the room. Usually the mosquitoes are kept alive for a 24 hour period to evaluate any knock-down effect of any intervention such as indoor residual spraying, if being deployed.

Figure 18. Hand collection of indoors resting mosquitoes

Exit trap collection (ETC)

This method involves fixing traps on the windows of bedrooms to determine the movement of mosquitoes during the night and their resting behavior (Fig. 19).

Figure 19. Exit trap

Essential materials: Flashlight/torchlight and batteries, exit trap cages, paper cups, aspirators/test tubes, forceps, cotton wool, filter paper, labels, 10% sugar solution.

The mosquitoes are caught from the inside of the traps, usually in the morning, and are placed into paper cups to be transported to the laboratory.

In the laboratory, female mosquitos are dissected to determine the physiological state of the abdomen. Young unfed mosquitoes suggest they were not successful in feeding. Blood fed mosquitoes suggest they were exiting the room to rest elsewhere to develop their ovaries. Gravid mosquitoes suggest they had developed their ovaries while resting indoors and are leaving to lay eggs outside.

6.2 Records of collection

The characteristics of each collection site should be described, namely:

- Locality and geographic location (GPS coordinates, name of the locality).
- Type of house and construction materials.
- Number of bedrooms and rooms in the house.
- Number of people that slept in the house the previous night and if they used a bednet (with or without insecticide).
- Type and general characteristics of the outdoor resting sites.

Information on the date and hour of the collections should also be noted. Mosquito samples should be labeled so that they can be identified according to the collection they belong. An example of a data collection form is given in Annex II.

There are other methods for collecting adult mosquitoes that used for particular situations. Some examples are Light Traps, Tent Traps, Colombian Curtains, and Double Nets. The student is encouraged to look up the references for more information.

6.3 Preservation of samples

Depending on the type of laboratory analysis to be undertaken with the collected mosquitoes, different preservation methods are used. The preservation methods are reviewed under Unit 7.

Unit 7
Preparation and Storage of Mosquito Samples

Learning objectives

This unit will focus on:

- **The main laboratory techniques used to analyze mosquito samples and purpose of the analyses.**
- **Describing which mosquito body parts are used in each technique, and how to prepare and store mosquito samples.**

Mosquito samples obtained from larval and adult surveys can be analyzed using a variety of laboratory techniques to obtain important information of the biology of the mosquito species and their role as a malaria vectors. Mosquito samples are generally used for:

- Morphological identification of species and species complexes to assess mosquito vector populations.
- Determination of the gonotrophic state to study resting behavior.
- Determination of physiological age and insemination of females to study the mosquito population longevity and survival.
- Detection of malaria parasites in the mosquitoes to determine sporozoite rates.
- Determination of the origin of the blood meal to study host preferences.
- Cytogenetic and molecular analyses for sibling species identification.
- Molecular analysis to study genes associated with insecticide resistance.

7.1 Main laboratory techniques

The following laboratory techniques are often used to analyze wild-caught mosquito samples from entomological surveys.

Species identification using morphological characteristics

In addition to the distinguishing anophelines from culicines, morphological structures can also be used to distinguish between anopheline species/species complexes (see also section 3.5 of Unit 3). This can be accomplished by the observation of species-specific characteristics, both at the immature and adult stages. Mosquitoes should be preserved in very good condition. Adults are kept dry inside entomological boxes with cotton wool and filter paper to avoid loss of scales during transportation. Larvae are preserved in tubes with 80% ethanol. The identification methods involve i) mounting specimens in slide and cover-slip (immature mosquitoes or adult body parts) for observation under a microscope; and ii) mounting adult specimens on a pin for

observation under a stereomicroscope. The identification involves the use of taxonomic keys. These identification techniques will be covered in an intermediate level course.

Mosquito dissections

Dissections are used to isolate certain internal organs of the mosquito female for observation usually under a microscope. These include:

- The spermatheca, to determine if the female has been inseminated.
- The ovaries, to assess the physiological age of the female (*e.g.* parity status).
- The stomach, to detect malaria parasite oocysts.
- The salivary glands, to detect malaria parasite sporozoites.

Mosquitoes to be dissected need to be fresh. Ideally, they should be killed or anaesthetized in the freezer immediately before the dissection. This implies transporting live mosquitoes from the field, either in paper-cups or in cages.

There are other more sophisticated techniques that provide answers to specific questions related to disease transmission vector biology. These are summarized in the following sections.

Enzyme-Linked Immunosorbent Assays (ELISA)

These immunochemical techniques use antibodies to detect specific proteins (antigens) of interest. In malaria entomology two ELISA methods are widely used:

- **Circumsporozoite-ELISA (CS-ELISA):** This assay detects the circumsporozoite (CS) protein that covers the outer surface of the malaria sporozoite and is thus an indicator of the presence of the infective stage of the malaria parasite. This protein starts to be expressed when the sporozoite is still in the mature oocyst on the midgut. Therefore, only heads and thoraces of mosquito females are analyzed to ensure that if CS is detected, it is most likely from sporozoites that reached the salivary glands and that the female is thus ready to inoculate malaria parasites. For this assay, mosquitoes can be kept dry at room temperature, inside tubes filled with silica gel and cotton.
- **Blood meal ELISA:** This assay is used to detect the origin of a blood meal that a female mosquito took before being collected. Antibodies against specific blood antigens of different hosts (*e.g.* human, cow, pig, dog, chicken) can be used in the ELISA to identify the blood source. The blood meal from the female (preferably freshly blood fed) is collected by squashing the abdomen onto filter paper. Blood spots are then kept dry at room temperature until the assay is done.

Cytogenetic analysis

This technique consists of the preparation of polytene chromosomes for the microscopic observation of banding patterns at the chromosomes that can be either species-specific or polymorphic. These polytene or "giant" chromosomes occur in the cells of only certain mosquito tissues/organs and only in certain life-stages or sexes. For example, in *An. gambiae*,

polytene chromosomes are found in the ovaries of half-gravid females or in the salivary glands of L4 larvae. Conservation of samples for cytogenetic analysis is normally done in Carnoy's solution (1 part acetic acid, 3 parts absolute ethanol), and kept at 4°C (fridge) or -20°C (freezer) for long periods of storage.

DNA/RNA-based molecular analysis

These techniques are usually used to differentiate members of sibling species complexes or to study genes of interest such as insecticide resistance associated genes. DNA-based techniques allow identifying genetic polymorphisms in genes of interest in mosquito populations. RNA-based techniques are used to study the levels of expression of these genes.

DNA is a very stable molecule which facilitates preservation of specimens for DNA extraction. Usually, mosquitoes are kept dry (in silica gel + cotton filled tubes) at room temperature or preserved in ethanol 80%. In addition, depending on the techniques, sufficient DNA for these studies can be obtained from very small mosquito parts (*e.g.* one leg).

RNA, on the other hand, is very unstable which makes the preservation of biological material more complicated, especially in field conditions. Ideally, mosquitoes should be killed and immediately stored in liquid nitrogen (-180°C) or at -80°C. The use of preservatives such as RNAlatter® allows for keeping the samples at room temperature or in the fridge, but only for a few hours or days.

For the conservation of mosquitoes for molecular studies, it is important to use a single tube to store each mosquito (or body part) in order to avoid contamination between specimens, as might happen if several mosquitoes were put together in the same tube.

7.2 Preparation of mosquito samples

When preparing mosquito samples for advanced laboratory analyses, different body parts of a single mosquito can be used for different techniques. For example, for a half gravid female, the head and thorax can be kept for CS-ELISA, the abdomen can be dissected to recover the ovaries for cytogenetic analysis and legs can be used for DNA extraction. Table 3 describes the mosquito body parts that can be used for different laboratory techniques and the way these are preserved.

Table 3. Type of preservation of mosquito body parts to be used in laboratory techniques

Technique	Biological material	Gonotrophic state	Conservation medium	Storing temperature
DNA extraction	Whole mosquitoes or body parts	Any	Adults: Silica gel and cotton. Larvae: Ethanol 80%	Room temperature (dry environment)
RNA extraction	Whole mosquitoes	Any	Liquid nitrogen *RNAlater*®.	-180°C (liquid nitrogen) -20°C or -80°C (*RNAlater*®)
Cytogenetics	Ovaries	Half-gravid	Carnoy's solution	4°C to -20°C
CS-ELISA	Head + Thorax	Any	Silica gel and cotton.	Room temperature (dry environment)
Blood meal ELISA	Abdomen (blood meal)	Fully blood fed	Whatman n°1 filter paper.	Room temperature (dry environment)

In order for these procedures to be successful, it is essential to adopt a good system for labeling and identifying the samples. All tubes (and filter papers) that contain body parts belonging to the same mosquito need to be labeled with the same number or sample code. Sample codes need to be informative, simple and unequivocal. The labeling is complemented with a database that describes every mosquito sampled.

During the sample processing it is very important to handle mosquitoes with care to avoid contamination between specimens. Use disposable materials whenever possible and sterilize the dissection equipment (forceps and needles) between specimens.

Label tubes containing liquid preservation solutions (e.g. ethanol, Carnoy's) with paper tags that are inserted inside the tubes and written with pencil. Tubes with silica gel can be labeled with permanent ink markers. Labels can be protected with transparent adhesive tape for protection.

7.3 Essential materials and equipment

Major equipment: stereomicroscope and optical microscope.

Materials: Entomological forceps and dissection needles, insect pins, plasticine, slides, coverslips, lamparine, cotton wool, plastic tubes (0.5ml, 1.5ml, 15ml), miscellaneous laboratory glassware, filter paper (Whatman n° 1), permanent ink markers, pencils, paper labels (parchment paper), adhesive tape, note book.

Reagents: Absolute Ethanol, distilled water, acetic acid, silica gel, mounting medium for slide preparations (*e.g.* entellan®).

7.4 Good laboratory practices

- Always keep the laboratory clean and organized.
- Wear protective masks and gloves when handling toxic or hazardous reagents. Read the safety brochure of the reagents before using.

- Clean and sterilize the dissection materials between specimens.
- Always use one tube for each part of the mosquito.
- Always label tubes in the same manner with readable handwriting.
- Correctly record the information on each mosquito processed and keep databases updated.
- Take good care of the optical equipment, such as microscopes.

Unit 8
Malaria Transmission Indices and Factors Affecting Transmission

Learning objectives

There are different malaria transmission patterns across different geographical areas. This unit will provide a basic understanding of:

- **The methods used to determine that a mosquito species is a malaria vector.**
- **Entomological indicators of transmission and how to calculate transmission indices.**
- **Some of the factors that affect malaria transmission.**

8.1 Determining which mosquito transmits malaria (vector incrimination)

In order to conclude that a species is a malaria vector, it is important to demonstrate that:

- Contact between the mosquito and humans does occur and that the mosquito feeds on human blood.
- There is a relationship, both in time and space, between the mosquito and the local cases of malaria.
- The salivary glands of the mosquito contain sporozoites (the stage of the malaria parasite that infects humans).

Several pieces of entomological information are needed to demonstrate the above, including:

- The presence and abundance of the mosquito.
- The feeding behavior of the vector: where and when a mosquito bites/feeds and source of the blood meal.
- Age or parity of the vector population.
- The percentage of mosquitoes of a given species that are infected with sporozoites.

The above information can be generated from longitudinal studies using sampling techniques, some of which were covered under Unit 6 and from which the following specific entomological indicators can be calculated:

- Vector resting habits
- Man-biting rate
- Longevity
- Sporozoite rate (infectivity)
- Human blood index

- Entomological inoculation rate (EIR)
- Vectorial capacity

8.2 Techniques for vector incrimination

Determination of blood digestion stage and ovarian development

Depending on the stage of blood digestion and egg development (*i.e.* the gonotrophic stage), the abdomen of the mosquito will assume certain coloration and shape (Fig. 20):

- *Unfed* – empty abdomen (no blood meal).
- *Freshly fed* – bright red with ovaries (white part) at the tip.
- *Half-gravid* – dark red color of blood covering 3-4 segments and ovaries/eggs covering the rest of abdomen.
- *Gravid* – blood absent or showing as a small black patch on the ventral surface of abdomen and the ovaries/eggs covering almost all of abdomen.

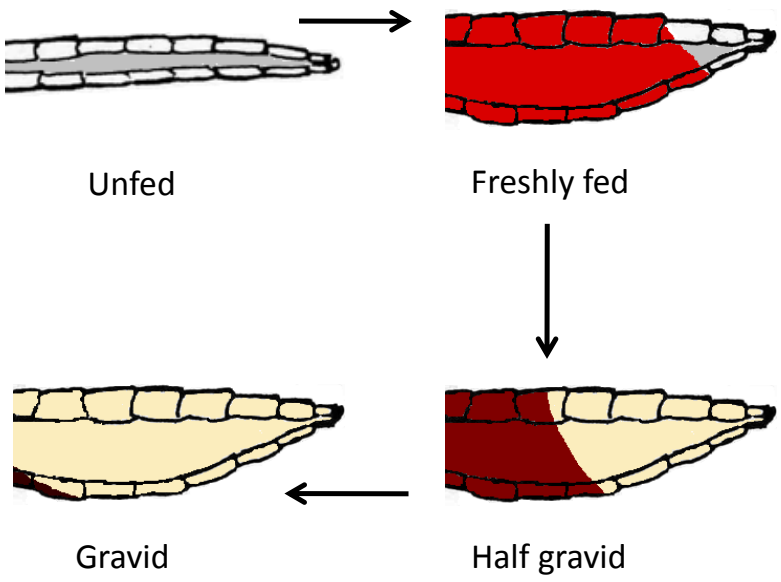


Figure 20. Abdominal condition of a female mosquito according to the gonotrophic state

Parity rate

The dissection of the abdomen and microscopic observation of the morphology of the ovaries can determine if:

- The female mosquito has laid eggs at least one time in her life – parous.

- If the female mosquito has not yet laid eggs – nulliparous.

This will allow estimating the parity rate of the mosquito population, *i.e.* the proportion of parous females, a parameter that reflects the age of the population. Older mosquito populations will have higher parity rates. Older populations are more likely to transmit malaria because they need to survive the time needed for the parasite to develop inside the mosquito and to take at least two blood meals to transmit the disease.

Infectivity of a population

The presence of sporozoites in the salivary glands of the mosquito indicates that the mosquito is able to transmit malaria parasites to humans. This can be determined through dissection and microscopic examination of mosquito salivary glands or by an ELISA technique. With this data, we can estimate the sporozoite (or infectivity) rate of a mosquito population.

Blood meal index and host preferences

An ELISA technique can also be used to determine the origin of the blood meal that a female mosquito has taken (*i.e.* whether the female has fed on a human host or other animal).

8.3 Estimation of transmission indices

This section is dedicated to understanding how to estimate some of the most important entomological indices used to characterize malaria transmission by a vector population. Some parameters, such as longevity or survivorship, require complex mathematical formulae, requiring additional training.

Indoor resting density (D)

Monthly indoor resting densities are calculated by:

- Collecting indoor resting mosquitoes in a number of selected houses in an area by PSC method. Usually the houses sampled are of equivalent sizes.
- Collected mosquitoes are then separated and counted by species and gender.
- The total female mosquitoes collected for each species is divided by the total number of houses sampled.
- Usually the sampling is done for two or three nights in a month and an average of the three nights is taken.
- Resting densities are thus expressed as the number of females per house per night. For example:
  - Assume that a total of 765 female mosquitoes of a given mosquito species were collected by PSC in four houses sampled in three consecutive nights of collection. Then,

Indoor Density (D) = (number of females ÷ number of houses) ÷ number of nights

D = (765 females ÷ 4 houses) ÷ 3 nights = 63.8 mosquitoes/house/night

Man-biting rate (ma)

The man-biting rate (ma) is expressed as the number of bites a person receives from a specific vector species per night. This parameter can be directly estimated from human landing catches by:

- Dividing the total number of mosquitoes caught by the total number of collectors.
- When collections are done during the whole night (*i.e.* 12 hours), the man-biting rate is expressed as number of bites per man per night:

  Man biting rate (ma) = number of mosquitoes collected ÷ number of collectors

- When collections are performed only for a few hours of the night, then the total number of mosquitoes caught should be divided by the total number of collectors and by the total time spent. For example:
  - Assume that five collectors captured 150 mosquitoes during four hours of collection. The man-biting rate would be:

Man biting rate (ma) = number of mosquitoes ÷ number of collectors ÷ number of collection hours

ma = 150 mosquitoes ÷ 5 collectors ÷ 4 hours = 7.5 bites/man/hour

An indirect way of calculating this parameter is through Pyrethrum Spray Collection (PSC) collections:

- All the freshly blood fed (**F**) females collected are separated by species and counted.
- The total number of collected females of a species is then divided by the total number of occupants (**W**) who spent the night in the rooms that were used for the collection. For example:
  - Assume that 63 freshly fed mosquitoes (**F**) were found in 3 houses (rooms) in one night and a total of 8 persons (**W)** slept in those 3 houses (rooms) during that night. Then:

ma = **F** ÷ **W** = 63 ÷ 8 = 7.9 bites per night.

The assumptions for this indirect estimation are that:

- All freshly fed mosquitoes obtained the blood from the occupants of the house that night.
- No freshly fed mosquito exited the house that night.

If the human blood index of the vector species is known, then the man-biting rate calculated from PSC can be adjusted by multiplying it by the value of the human blood index.

Endophagic and Exophagic indices for biting preference

The endophagic and exophagic indices can be directly obtained from man-biting rate estimates calculated from human-baited landing catches. This involves:

- Conducting landing catches with collector teams placed indoors and outdoors at the same time.
- Calculating indoor and outdoor man-biting rates.
- Calculating the endophagic index as the proportion of females of a given species that bite indoors (the exophagic index being the proportion of females that bite outdoors). For example:
  - Assume that in four hours of landing catches, two collectors captured 168 female *An. gambiae* s.l. inside bedrooms and at the same time two other collectors captured 122 female *An. gambiae* s.l. outside in the village. Then:

    1. The indoor man-biting rate is:

ma(i)=168 females ÷ 2 indoor collectors ÷ 4 collection hours = 21.0 bites/man/hour

    2. The outdoor man-biting rate is:

ma(o)=122 females ÷ 2 outdoor collectors ÷ 4 collection hours = 15.3 bites/man/hour

    3. The endophagic index (ENGI) is then:

ENGI = ma(i) ÷ [ma(i) + ma(o)] = 21.0 ÷ (21.0 + 15.3) = 0.58

    4. The exophagic índex (EXGI) is then:

EXGI = ma(o) ÷ [ma(o) + ma(i)] = 15.3÷ (15.3 + 21.0) = 0.42

Note that ENGI+EXGI always equal 1.

Sporozoite rate(s)

The sporozoite rate is the proportion of mosquitoes of a given species that were found to carry sporozoites in the salivary glands (either by dissection or CS-ELISA). For example:

- Assume that 1500 female anophelines of the same species are analyzed by CS-ELISA. Of these, 32 were found positive for circumsporozoite protein (an indicator of the presence of sporozoites). Then:

Sporozoite rate (s) = number of positive mosquitoes ÷ number of analyzed mosquitoes

s = 32 positive mosquitoes ÷ 1500 analyzed mosquitoes = 0.021 (or 2.1%)

Human Blood Index (HBI)

The HBI can be obtained from analyzing the blood meals, usually by ELISA techniques, from identified species caught in the field by resting collections. The HBI is then calculated as the proportion of females of a given species that were verified with human blood in their stomachs. Similar indices can be calculated for any blood meal type present in the analyzed collection. For example:

- Assume that blood meal analysis by ELISA of a given *Anopheles* species revealed that 83 fed on humans, 11 fed on chickens and 36 females fed on dogs. Then:

HBI = n° human feeds ÷ (n° human feeds + n° chicken feeds + n° dog feeds)

$$HBI = 83 \div (83 + 11 + 36) = 0.63$$

Determining vector resting habit following a blood meal

Determining where the vector rests after a blood meal is very important for evaluating the potential of an intervention tool (such as indoor residual spraying) in disrupting transmission. The post-feeding resting habit (f) can be calculated using the other parameters that have been described above:

$$f = [k \times H \times D] \div [N \times P \times M]$$

Where:

k = a correction constant of 1.16.
H = HBI estimate.
D = indoor resting density calculated from PSC collection.
N = average number of persons per house.
P = duration of indoor resting after blood feeding. This parameter is obtained from the analysis of the abdominal condition of resting females. P = 1 + (number of half-gravid and gravid females ÷ number of freshly fed females).
M = monthly man-biting rate estimate.

Entomological Inoculation Rate (EIR)

While there are more complicated methodologies for calculating malaria transmission, a simpler way is to calculate the entomological inoculation rate (EIR), which is defined as the number of infective bites received per person per night. EIR may be calculated using the parameters defined in the previous sections:

EIR = [Man-biting rate (ma)] x [sporozoite rate (s)]

For example, assume that for an identified species A, the man-biting rate was determined as ma = 7.9 and the sporozoite rate was s = 0.003. Then:

EIR = ma x s = 7.9 x 0.003 = 0.02 infective bites/person/night

This means that, in a month of 31 days, 0.62 infective bites (0.02 x 31 days) are anticipated from species A. Likewise, the annual EIR for this species should be around 7 (0.02 x 365 days) infective bites per year.

8.4 Factors affecting malaria transmission

The intensity of malaria transmission by malaria vectors is affected by environmental and anthropogenic/demographic factors. The environmental factors seem to impact differently on different species. The main environmental factors affecting malaria transmission include:

Rainfall: In subtropical and tropical regions, variation in rainfall accounts for the seasonality of most mosquito species. Rainfall creates the temporary breeding sites that are critical for the surge in population density of some vector species and resultant spikes in transmission. For example, there is generally a positive correlation between rainfall and the adult density or clustering of *An. arabiensis* and *An. gambiae* s.s. Rainfall is however negatively correlated with clustering of *An. funestus*.

Temperature and humidity: While in temperate regions temperature is the main determinant factor influencing the population dynamics of anophelines, this effect appears to be comparatively less evident in tropical climates. *Anopheles* mosquitoes become inactive at cold temperatures. Colder water temperatures can slow down larval development and emergence. However, mosquito longevity significantly decreases at temperatures above 35°C and relative humidity below 50%. Mated females of *An. gambiae* can survive hot and dry long periods by aestivation. Likewise, some mosquito species from temperate regions are known to hibernate during winter.

Altitude: It is known that malaria transmission generally tends to fall with increasing elevation. Anophelines are not generally found at altitudes above 2,000 meters. Temperature decreases on average 6.5°C for every 1000m and this decrease will slow down the development of the malaria parasite inside the mosquito, thus influencing malaria transmission.

Anthropogenic/demographic factors: These include housing type, human activities that promote availability of breeding sites, poverty, and behaviors linked with the level of understanding of malaria transmission risks, as well as socio-cultural practices.

Unit 9
Essentials of Rearing Mosquito Colonies in the Laboratory

Learning objectives

This unit will provide knowledge on:

- **The basic characteristics of an insectary.**
- **Basic requirements for rearing larvae and adult anopheline mosquitoes in a laboratory environment.**

9.1 The insectary and basic operations

An insectary is a place where insects are raised (reared) and maintained under laboratory conditions. It may range between sophisticated and simple, depending on the purpose for which it is set up. For the purpose of routine vector control, an insectary can be relatively inexpensive.

Insectaries are important for maintaining an adequate supply of mosquitoes for observation, identification and various assessments, such as susceptibility assays to insecticides, estimation of mosquito longevity and feeding habits.

It may consist of a small room for maintaining larvae and adult colonies (Fig. 21), or preferably two rooms; one each for larvae and the adult colonies.

Figure 21. Image of an insectary showing larval trays and adult cages

Sometimes an insectary may contain colonies of fully susceptible, as well as wild-caught (local) species. In such situations it is essential that the species (especially the susceptible species) do not become contaminated with wild species.

An insectary should be established with a central goal of preventing escape or self-entry of mosquitoes. It will usually have a low ceiling (not more that 7ft or 220cm), cement floors and walls with light paint (white or off-white walls). These characteristics are necessary for spotting escaped specimens. Doors and windows should be screened. In addition:

- There should be adequate security to prevent entry of unauthorized persons.
- Furniture should be in rust-proof metal, fiberglass, plastic or at least clean polished wood. Legs of furniture should be isolated from the floor (usually using containers filled with oil) and from the walls to prevent invasion by ants and other crawling insects.

Knowledge of the specific requirements of the survival of the mosquito vector species (temperature, food, humidity and light) is critical for successful rearing in the lab.

Take extreme care to prevent microbial growth as this will severely threaten the survival of the insect colonies. An insectary should be clean and uncluttered. Ensure that no insecticide or chemical is introduced into the insectary.

Take care to prevent the insect food from growing moldy; refrigerate larval food and prepare an appropriate amount as needed. Sugar water for adults is particularly prone to microbial growth and should be changed regularly.

Regular pest control, particularly targeting ants and cockroaches, is important as these tend to feed on the mosquito colonies.

It is important to maintain a constant schedule of specific tasks (*e.g.* when to feed larvae, when to collect eggs, when to blood feed adult mosquitoes). A good practice will be to develop and pin the schedule in an easily accessible place or notebook.

Maintaining genetic purity of insect colonies is extremely important. Once an insect stock is contaminated, then it is of little value. Therefore, it is important to prevent cross-contamination of eggs, larva and free flying adults in the insectary. These are all sources of stock contamination.

Maintaining proper nutrition is essential for the survival and fecundity of the mosquito colony. Optimization of the nutrition, photoperiod, competition (i.e. larval and adult densities inside trays and cages, respectively), and temperature will result in a more productive colony.

9.2 General conditions for rearing mosquitoes

Egg

Only a few *Anopheles* species are easily reared in the insectary. For those species that can be reared, there are two ways to start a colony with wild local mosquitoes. A colony can either be

started from captured resting adult females that have already taken a blood meal and that are kept fed on sugar solution until they lay eggs; or from collection of larvae from the wild, raising them to adults and then producing a first batch of eggs by blood feeding the grown females. The steps are:

- Collect larvae or blood fed indoor resting females from the field and put them into a mosquito cage in the insectary.
- Maintain them in the insectary with stable relative humidity (80%) and temperature (27°C).
- Maintain adults on 10% sugar solution.
- To get adults to lay eggs, it may be necessary to blood feed them (in case of adults raised from larvae in the insectary).
- Put egg-laying dishes inside the cages to capture eggs. An egg-laying dish can be easily made using the top or bottom parts of a Petri dish, which is filled with a little water and a piece of filter paper fit to the dish. (or something like that)

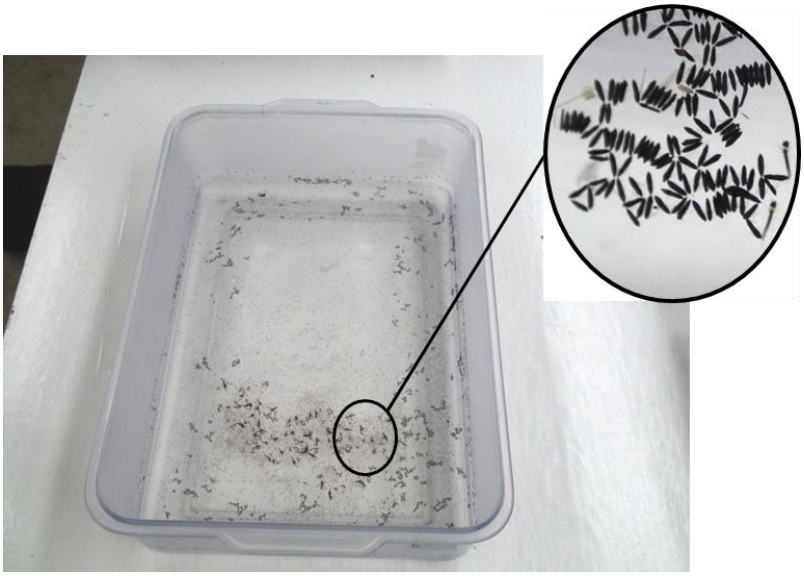


Figure 22. Larval tray with eggs and 1st instar larvae

Basic egg hatching procedure

- Cover base of larval tray with de-ionized water and add yeast solution to final dissolved concentration of 0.02% (e.g. 300ml water and 3ml of a 2% w/v yeast solution).
- Gently rinse eggs into tray, cover the tray and leave undisturbed for 24 hours ensuring that the eggs do not stick to the sides of the tray above the solution.
- Eggs hatch between 24-48 hrs. Inspect for first instar larvae in bright light (Fig. 22).

Larvae

Temperature is the most important external factor that affects the growth rate of larvae. A constant water temperature of about 27°C is critical for larval development.

The larva room should have a wide glass window to let in daylight or an artificial lighting system that allows for alternation of 12-hour light/dark cycles, another critical environmental factor for larval development.

Larvae should be fed twice daily, usually with ground fish food. Both the quality and quantity of the diet are important to the longevity and fecundity of the adult stage. Larval mortality can be high if they are overfed. Underfeeding will produce smaller adults. The first feeding should be 24 hrs after hatching. The first instar larvae require more food than the fourth instar.

Check for algae inside the larval trays as these can lead to high mortality. It is important to change the rearing water every 1-2 days and remove all dead larvae in the trays. Larvae should be handled gently, especially during transfer from tray to tray.

Larva trays should not be overcrowded, as this will affect the larval development due to competition for food and cannibalism.

Pupa

This is a non-feeding stage. The larval trays should be checked daily for pupae. Pupae are removed and placed into adult cages where the adults will emerge. Separation of pupae from larvae can be done simply by the use of a pipette or a fine-net spatula; pupae are placed into a cup for transfer into an insect cage (Fig. 23). For larger numbers, the tendency for larvae to swim down when disturbed, whereas pupae are tends to stay up can be used to facilitate this procedure. A cup containing larvae and pupa can be whirled and this causes the larvae move to the middle bottom of the cup but the pupa will stay up on the side. They can be separated then by use of a pipette. Chilled water can also be used to reduce pupal and larval activity to facilitate separation (but needs to be done quickly to avoid harm to developing stages) which then enables the pupae to be poured out into a sieve and transferred to water at room temperature.

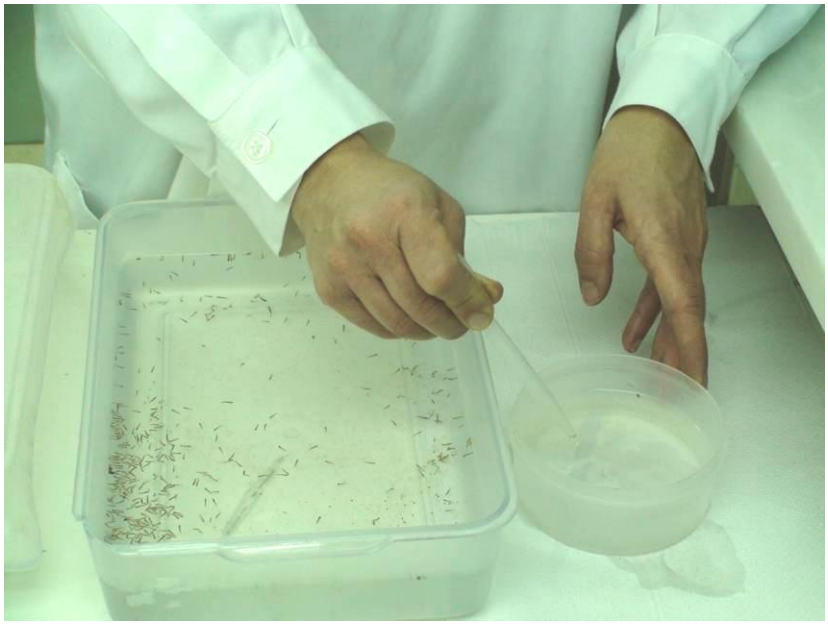


Figure 23. Separating pupae from larvae with a pipette

Adults

Adults can be kept in net covered cups, which are inexpensive and can hold about 10-15 adults. Usually a hole is made in the net to introduce the adults and plugged with cotton wool. Adult cages can be used to hold larger numbers. Adults should be handled with care, using mouth aspirators from transfers between cages whenever necessary.

Adult diets have been shown to affect longevity and fecundity. Those fed only on blood have been shown to live longer than those fed on a combination of sugar and blood. Generally, females should be given blood meal every 2-3 days. Laboratory animals used for feeding adults (e.g. rabbits, guinea pigs) need to be kept away from the insectary. Always check the cotton wool with 10% sugar solution in the cage and replace the cotton every day.

A constant relative humidity of 80% (±10%) is critical for adult survival. This can be achieved through the use of steam injectors, humidifiers or evaporative coolers. Temperature should also be kept stable, at 25°C-27°C.

Unit 10
Insecticide Susceptibility and Cone Bioassay Tests

Learning objectives

Resistance of mosquito vectors to insecticides that are used in their control is a growing problem globally that threatens the sustainability of malaria control programs. At the end of the unit, the student will know how to conduct:

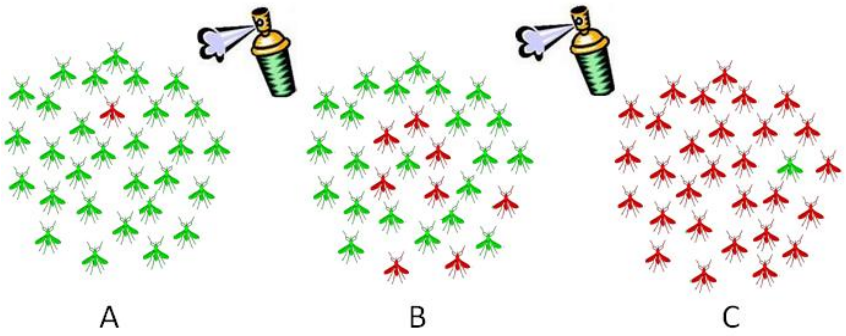
- **The WHO test to estimate the susceptibility of vectors to insecticides.**
- **The WHO bioassay to assess residual efficacy of insecticide on sprayed surfaces.**
- **The WHO bioassay to assess residual efficacy of long lasting insecticidal nets.**

10.1 Why determine the susceptibility of malaria vectors to insecticides?

If a vector is susceptible to an insecticide, then it means that the vector will be killed when it comes into contact with the insecticide at the prescribed dosage that is used for the particular intervention (indoor residual spray, insecticide-treated bed net or larvicide). Decreasing susceptibility means that the vector becomes increasingly tolerant to the insecticide, up to a point where it becomes resistant.

If a vector develops resistance to an insecticide, it means it can withstand the dose that normally would have killed it and this may undermine the effectiveness of the intervention. It is therefore important to know the susceptibility level of the local vector to the insecticides to be used in the intervention.

Insecticide resistance comes about as a result of the interaction of selection pressure, genetic variability (mutation), gene flow and the life history of the mosquito population (Fig. 24).



A. Genetic mutations that confer insecticide resistance to mosquitoes, generally occur at a very low rate in natural populations; B: under insecticide selective pressure, mutants will survive better and wild-type (susceptible) mosquitoes will die. C: after several generations of continued insecticide pressure, mutant resistant mosquitoes will prevail in the populations.

Figure 24. Selection of insecticide resistance in vector populations

10.2 Preparation of test vectors for susceptibility and cone bioassay evaluations

Two general methods are used to prepare/obtain test vectors for bioassays:

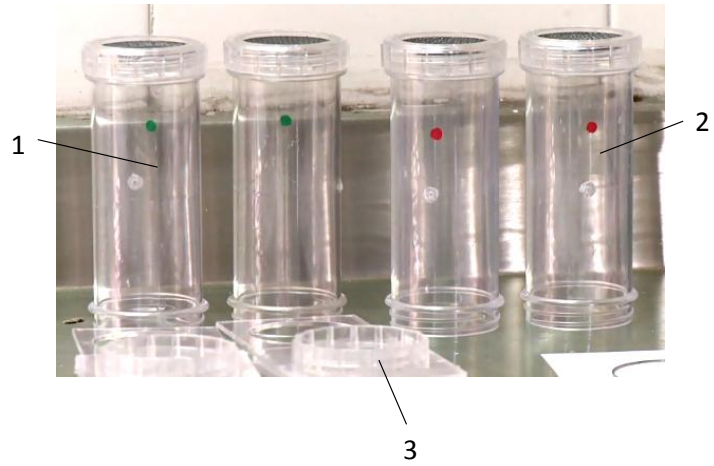
- Larvae may be collected from a range of local breeding sites (see Unit 5). The larvae are then reared under laboratory conditions (see Unit 9). Pupae are then transferred daily into adult cages to capture emerging adults. The adults are then fed on ten percent sugar solution and kept in cohorts of 3-5 days after emergence.
- Alternatively, blood fed and gravid local mosquito species are collected using adult sampling techniques described under Unit 6. These are then kept on 10% sugar solution to lay eggs (see Unit 9) and the resulting F1 generation is reared to 3-5 day old adults to be used in the tests. In such cases a minimum of about 50 females are used to provide eggs to ensure adequate genetic variability. It is often quite difficult to obtain large numbers of ovipositing females (*i.e.* females who actually lay eggs in captivity) with *An. funestus* and *An. darlingi*.

10.3 Determining the susceptibility of adult mosquitoes

There are two standardized methods for determining vector susceptibility to insecticides in adult mosquitoes. Note that these methods only measure a reduction in insecticide susceptibility in vector populations. They are not a direct measure of resistance. To confirm resistance, additional analysis is required to determine the mechanisms underlying reduced susceptibility.

CDC bottle bioassay: This method is widely used in several countries and the protocol may be accessed at www.cdc.gov/ncidod/wbt/resistance/assay/bottle/index.htm .

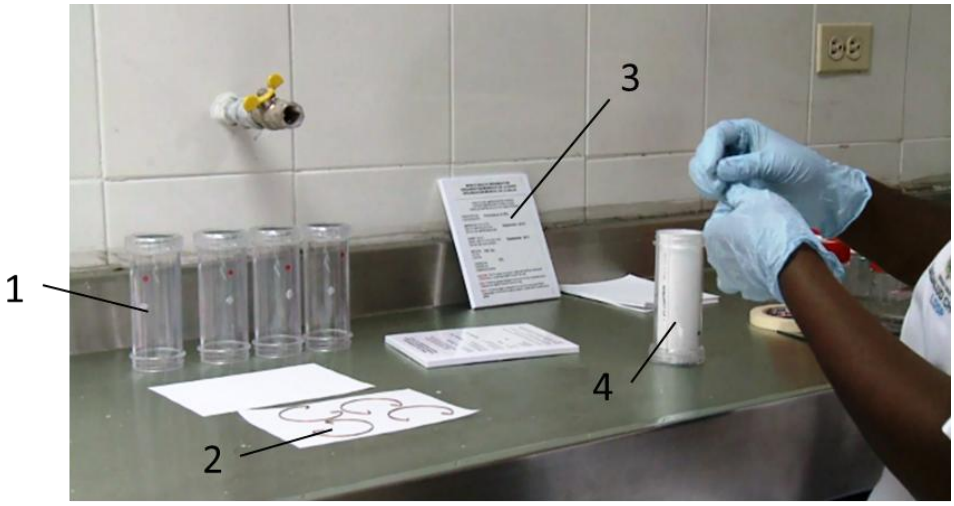
WHO Tube Assay: The standardized methodology is provided by the World Health Organization (WHO) for assessing the susceptibility of female *Anopheles* (WHO 1998). Mosquito vectors of a known species are exposed in special test tubes containing filter papers, impregnated with a lethal concentration (discriminating dose) of a given insecticide dissolved in oil.



1. Control/holding tubes (green dot), 2. exposure tubes (red dot), 3. slide units

Figure 25. WHO test tubes for susceptibility testing

The WHO tube test kit is made up of two plastic tubes that are 44 mm in diameter and 125 mm long (Fig. 25). One end of each tube has a 16-mesh screen. One of the tubes is marked with a red dot and is used as "exposure tube" as it is lined with insecticide impregnated filter paper held in place with two copper clips. The other tube, with a green dot, serves as a "holding tube", which has its inner walls lined with plain paper held in place by two steel clips (Fig. 26).



1. exposure tubes (red dot), 2. clips, 3. box with impregnated papers, 4. control tube (green dot)

Figure 26. Lining the test tubes with impregnated papers

The holding tube is attached to a slide unit which has a 20 mm hole through which mosquitoes are introduced using an aspirator. The exposure tube can be screwed to the other side of the slide unit. A sliding partition within the unit opens a hole between the tubes and the test mosquitoes are gently blown into the exposure tube to start the exposure to the insecticide and then blown back to the holding tube after a timed exposure.

In each assay, another exposure tube (also marker with a green dot) is lined with a filter paper impregnated only with the oil used to dissolve the insecticide. This exposure serves as control in the assay. The steps of the assay are:

- Connect the holding tube with slide unit.
- Transfer 15-25 mosquitoes to each of the holding tubes through the connecting hole. The sugar fed test mosquitoes are carefully collected from the adult cages using an aspirator. Leave the mosquitoes to rest in the holding tubes for 60 min.
- Connect the exposure tubes (include that of the control) to each of the holding tubes on the other end of the slide unit. Open the slide unit and blow gently the mosquitoes from the holding tube to the exposure tube.
- Close the slide, disconnect the holding tubes and allow the exposure (and control) tubes to stand upright for the one hour (two hours if using fenitrothion). Note any dead mosquitoes at 15minutes intervals during the exposure stage.
- After the exposure period, transfer the mosquitoes back to the holding tube, and let it stand upright for 24 hours. Place a piece of moist cotton wool on the wire mesh end of the tube and place the tubes in a wooden box with large holes for ventilation and covered with a damp towel. The temperature and humidity in the box should be monitored (25±2°C; 75±10%).

- 24 hours after exposure, count the dead mosquitoes killed by the contact with the insecticide and those killed in the control.
- If 25 mosquitoes per tube is used, then a minimum of 100 mosquitoes in four to five replicates need to be tested to calculate percent mortality of one insecticide.
- The mortality rates are calculated as:
  - **Control mortality:** C = (n° of dead mosquitoes)/(n° of mosquitoes tested) in control tube.
  - **Exposure mortality**: E = (n° of dead mosquitoes)/(n° of mosquitoes tested) in the exposure tubes with insecticide.
  - If control mortality is between 5% and 20%, the value for exposure mortality, E, is corrected by Abbott's formula:

Corrected exposure mortality: E' = [(E- C) / (100-C)] x 100

Where E is the (uncorrected) exposure mortality expressed in percentage and C is the control mortality expressed in percentage.

For instance, if control mortality C is 10% and crude exposure mortality E is 40%, the corrected exposure mortality is [(40 – 10)/100 – 10)] * 100 = 33% .

- If control mortality is lower than 5%, there is no need for correcting E.
- If control mortality is greater than 20%, the experiment should be discarded.

Interpretation of results

- Mortality rate between 98% and 100% indicates susceptibility
- Mortality rate between 80–97% suggests possible resistance. It is desirable to undertake additional wider sampling and testing to reconfirm levels in the vector population.
- Mortality rate lower than 80% indicates resistance

Biochemical analysis or PCR methods can be used to identify mechanisms underlying the development of the resistance detected. This will enable effective resistance management measures to be implemented.

10.4 Residual Efficacy of Insecticide on Sprayed Surfaces (WHO 1998, 2005)

The residual efficacy of an insecticide on a sprayed surface is determined by cone bioassay tests. This is done by checking mortality of the target mosquito vector species exposed to the sprayed surface at intervals of weeks or months after the spraying. This technique can be also used to evaluate the quality of a residual spraying operation. It is also used to determine residual efficacy of an insecticide on bed nets.

The WHO cone bioassay kit includes plastic cones, adhesive sponge tape, bent aspirator or sucking tube, normal aspirators or sucking tubes, cardboard paper, small nails, hammer, cotton

wool, paper cups with cover nets, rubber bands, markers, mosquito cage, wooden box with large holes, towels. The plastic cones are shown in Fig. 27.

Figure 27. WHO cone bioassay on a wall

The procedure of the wall assay is:

- Line the edges of the plastic cone with adhesive sponge tape.
- The cone should be fixed on the sprayed surface using scotch tape or nails. Cones are fixed at three different heights (low, middle and high).
- Insecticide-free cardboard paper is nailed into the wall on top of which plastic cones are fixed and used as control.
- 10 mosquitoes from a fully susceptible *Anopheles* strain from an insectary are introduced into each cone and a piece of cotton wool inserted in the opening of the cone. Use a separate aspirator/sucking tube to transfer mosquitoes to control cones.
- After a specified exposure period (usually 30 minutes), carefully take out the mosquitoes from the cones and transfer them to separate (and labeled) paper cups. Count the number of mosquitoes dead or "knocked down" at the end of the exposure period but do not remove them, as some of them might later recover.
- Place a damp cotton wool on the top of the paper cups, put them in the wooden box and cover with a damp towel.
- After 24 hours, count the number of dead mosquitoes and calculate the percent mortality in both exposure and control paper cups.
- If mortality at the control is between 5% and 20%, the exposure mortality should be corrected by using Abbott's formula described above. Discard the results if mortality is greater than 20%.
- For each wall type tested, the experiment should be repeated on different walls within the same house and also in different houses to have a representative sample.

Residual efficacy of insecticide on bed nets

The bioassay procedure for insecticide-treated bed nets is similar to the procedure used for sprayed walls, except that the mosquitoes are exposed only for three minutes in batches of 5 mosquitoes per cone. Two cones are placed in each side and on the top of the bed net, at different heights. As control, two cones are applied to a non-treated bed net. Exposure cones can be attached to the net using rubber bands (Fig. 28). The test can also be done by taking the net down and stretching a section over a piece of cardboard.

Figure 28. WHO cone bioassay on an insecticide treated bednet

Annex I Sample of Training Curriculum and Schedule for a Basic Entomology Technician Course

Introduction

Malaria remains a major cause of sickness and child mortality in tropical regions of the world. This disease imposes severe constrains on economic development, being an important cause of poverty in most disease endemic countries. Although there has been a sharp increase in funding for malaria control, disease reduction targets set by the Roll Back Malaria and National Malaria Control Programs still remain unachieved in many countries. This is largely due to the lack of capacity in generating adequate knowledge on local disease eco-epidemiology to inform program implementation and management. In particular, capacity for entomological monitoring and surveillance is still rudimentary. There is an urgent need for National Malaria Control Programs do create adequate numbers of trained personnel to effectively participate in malaria control activities.

Objective of the training

The course aims at supporting efforts by the national malaria control program (NMCP) to build a critical mass of trained staff, at regional level, for entomology surveillance and monitoring to guide malaria vector control interventions. The training will provide entomology technicians with basic knowledge on the role of vector control in malaria control, the biology and control of mosquito vectors, as well as competency in standardized methodologies for the surveillance and monitoring of malaria vectors.

Target audience

The course targets district level personnel in malaria endemic countries who will normally form the cadres that collect and report on local entomological indicators in support of vector control programs. They will normally have secondary level education or diploma in a subject area that lends itself to training in entomology.

Course structure

The course is organized into 10 learning units. Each learning unit includes theoretical, practical and theoretical-practical (T-P) classes. The course is designed for three weeks but it can be shortened to two-weeks depending on the needs of the country. The first two weeks are devoted to the learning units. Theoretical classes will be concentrated in the first week and practical and T-P classes in the second week. The third week will be dedicated to demonstrations of vector control tools and methods, revision and general discussion classes, students and course evaluations.

Course evaluation

The course will begin with a pre-test that will enable a refinement of the scope of the course to better meet the training objective. At the end of the course, students will reply to a final test to

assess the acquisition of new knowledge and to a confidential questionnaire to evaluate the quality of the course in its different components.

Course contents

All the areas to be covered by the training are outlined in-depth in the *Manual for Entomology Technicians training* (basic level). The manual will serve as the basic document guiding the training. The purpose and main contents of each learning unit are presented in Table A-1.

Course schedule

A proposed schedule for the 3-week basic course is shown in Table A-2. This schedule is flexible and will depend on the logistics of the venue and the requirements of the course receptors (national programs).

Table A-1. Objective and contents of learning units

Unit	Title	Objective	Contents	Classes
1	Malaria control and the role of entomology	At the end of this unit, students will be able to identify the role of vector control in malaria control programs, the main tools available for vector control and the basic principles for its implementation	• Basic approaches to malaria control • Vector control tools • Vector control and principles for effective implementation • Basic principles of planning malaria vector control	Theoretical classes Practical classes: demonstration of main vector control tools (bednets, residual spraying, larvicides)
2	Biology of malaria vectors	At the end of this unit, students will have learnt the major features in the life-cycle of a mosquito and the main biological, ecological and behavioral traits that influence the capacity of a mosquito to transmit malaria.	• Life-cycle of the *Anopheles* mosquito • Main bio-ecological traits of medical importance	Theoretical classes Practical classes: demonstration of the different stages of the life-cycle in the insectary
3	Mosquito anatomy and identification	At the end of this unit, students will be capable of morphologically distinguish *Anopheles* mosquitoes, which are vectors of human malaria, from culicine mosquitoes, which do not transmit human malaria.	• Main morphological characters of mosquitoes (Diptera: Culicidae) • Distinguishing between *Anopheles* (subfamily Anophelinae) and culicines (subfamily Culicinae) at the immature and adult stages	Theoretical classes Practical classes: Identification of the major distinguishing characteristics of mosquitoes, morphological identification of *Anopheles* sp.
4	Diversity of malaria vectors	In most endemic regions, malaria transmission is sustained by several mosquito vector species and, in some occasions, different subpopulations. At the end of this unit, students will have acquired information on the complexity of vectorial systems in nature.	• Geographic distribution of malaria vectors, habitat diversity and ecological adaptation • Concept of sibling species complex • Description of the *Anopheles gambiae* complex and the *Anopheles funestus* group	Theoretical classes Practical classes: Introduction to taxonomic keys and its use for adults and larvae
5	Mosquito surveillance (Larvae)	At the end of this unit, students will be capacitated to conduct larval sampling within the framework of entomological surveys for mosquito monitoring.	• Reasons for larval studies • Range of larval habitats and factors affecting adult production from aquatic habitats • Sampling methods, preservation of samples and processing • Environmental factors commonly recorded • Data handling and analysis	Theoretical classes Practical classes: Sampling and identification of mosquito larvae and pupae
6	Mosquito surveillance (Adult)	At the end of this unit, students will be capacitated to conduct adult sampling within the framework of entomological surveys for mosquito monitoring.	• Types of mosquito surveys • Adult mosquito collection methods (night landing collection; pyrethrum spray sheet collection; outdoor resting collection; exit-trap collection; hand collection/aspiration)	Theoretical classes Practical classes: Adult sampling using different techniques and processing of samples
7	Preparation and storage of mosquito samples	At the end of this unit, students will be able to decide about and apply the correct methods for handling and storing mosquito samples to be used in subsequent laboratorial analyses.	• Main laboratory techniques used in malaria entomology (morphological identification, dissections, ELISA, cytogenetics, DNA/RNA-based molecular analysis) • Preparation of mosquito samples for the different techniques • Essential materials and equipment	Theoretical classes Practical classes: preparation of mosquito samples for blood meal ELISA and DNA-based analysis.

Unit	Title	Objective	Contents	Classes
8	Malaria Transmission indices and factors affecting transmission	At the end of this unit, students will have knowledge about the major factors that affect malaria transmission. They will have learnt how to obtain biological information to incriminate vectors and calculate transmission indexes. They will also be able to calculate and interpret basic entomological and epidemiological indexes used to establish transmission levels	• Determining which mosquito transmits malaria (vector incrimination) • Selected vector incrimination techniques • Estimation of transmission-related indices, including indoor resting densities, human biting rates, host preference; vector resting habit following a blood meal; indoor and outdoor biting preferences; entomological inoculation rates (EIRs) • Factors affecting malaria transmission	Theoretical classes Practical classes: determination of blood digestion and ovarian development stage; demonstration of mosquito dissection and identification of salivary glands and ovaries T-P classes: calculation of transmission indices using results from field sampling
9	Essentials of rearing mosquito colonies in the laboratory	At the end of this unit, students will have acquired information about the requirements of an insectary and the major steps to be performed to establish and maintain mosquito colonies, as well as their usefulness for malaria vector monitoring.	• Insectary and basic operations • General conditions for rearing mosquitoes: egg, larva, pupa and adult	Theoretical classes Practical classes: Field collection of wild mosquitoes for establishing a colony in a basic insectary
10	Insecticide susceptibility and wall bioassay test	At the end of this unit, student will be capacitated to conduct bioassays for determining the insecticide susceptibility of mosquito populations and to carry out bioassays to assess the residual efficacy of insecticide-treated surfaces (walls and bednets).	• Reasons for determining susceptibility of vectors and residual efficacy of insecticides • WHO test: susceptibility of adult mosquitoes to insecticides • WHO test: residual efficacy of insecticide on sprayed surfaces • CDC susceptibility bottle-assay	Theoretical classes Practical classes: field demonstration and practice of susceptibility tests and wall bioassay

Table A-2. Course schedule (week 1)

Time	Day 1	Day 2	Day 3	Day 4	Day 5
9:00 AM	Opening Ceremony	Major Vector Control Interventions (Unit 1; T)	Vector Incrimination (Unit 8; T)	Preparation and storage of mosquito samples (Unit 7; T)	Data and Interpretation
9:30 AM					
10:00 AM	Course Objectives				
10:30 AM	*Coffee Break*	*Coffee Break*	*Coffee Break*	*Coffee Break*	*Coffee Break*
11:00 AM		Basic Principles of Planning Malaria Control (Unit 1; T)	Mosquito surveys (larvae) (Unit 5; T)	Rationale for Determining Insecticide Susceptibility (Unit 10; T)	Establishment & Maintenance of Mosquito Colonies (Unit 9, T)
11:30 AM	Pre-test				
12:00 PM	*Lunch*	*Lunch*	*Lunch*	*Lunch*	
12:30 PM					*Lunch*
1:00 PM	Biology of Malaria Vectors (Unit 2; T)	Mosquito Identification (Unit 3; T)	Mosquito Surveys (Adults) (Unit 6; T)	Type of Tests Available from WHO & CDC (Unit 10; T)	
1:30 PM					Collection and Transportation of Larvae & Adult Mosquitoes for Insectary (Unit 9, P)
2:00 PM				Practical of WHO Bioassays, Cone Tests and/or CDC Bottle Assays (Unit 10, P)	
2:30 PM	Basic principles of malaria control (Unit 1; T)	Diversity of Malaria Vectors (Unit 4; T)			
3:00 PM					*Coffee Break*
3:30 PM	*Coffee Break*	*Coffee Break*	*Coffee Break*	*Coffee Break*	Standard Operating Procedures for Colony Maintenance (Unit 9, T-P)
4:00 PM	The role of entomology in malaria vector control (Unit 1; T)	Diversity of Malaria Vectors (Unit 4; T)	Estimation of Malaria Transmission Parameters (Unit 8; T)	Practical of WHO Bioassays, Cone Tests and/or CDC Bottle Assays (Unit 10, P)	
4:30 PM					
5:00 PM					

T: theoretical class; T-P: theoretical-practical class; P: practical

Table A-2. Course schedule (week 2)

Time	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
6:00 AM		Field Trip (Identify breeding places and larval sampling) (Unit 5, P)		Pyrethrum Spray Collection (Unit 6, P)		
8:00 AM						
8:30 AM				Processing of Mosquitoes (Units 3&7, P)		
9:00 AM	Organizing field activities & the essentials (Units 5 & 6, T-P)		Processing of Samples from NBC and LTC (Units 3&7, P)		Larval survey (Unit 5, P)	Processing of Samples from NBC and LTC (Units 3&7, P)
9:30 AM						
10:00AM				ORC & exit trap collections (Unit 6, P)		
10:30AM	*Coffee Break*					*Coffee Break*
11:00 AM	Breeding Habits and Larval Control (Units 1&2, T)	Mosquito identification (larvae) (Unit 3, P)				Processing of Samples from NBC and LTC (Units 3&7, P)
11:30 AM				*Lunch*		
12:00 PM	*Lunch*		*Lunch*			
12:30 PM		*Lunch*			*Lunch*	*Lunch*
1:00 PM	Data reporting management, and utilization (Units 5&6, T)		Calculation of Malaria Transmission Parameters (Unit 8, P)			
1:30 PM		Mosquito identification (adult) (Unit 3, P)		Processing of Mosquitoes (Units 3&7, P)	Mosquito identification (larvae) (Unit 3, P)	
2:00 PM						Calculation of Malaria Transmission Parameters (Unit 8, P)
2:30 PM						
3:00 PM	*Coffee Break*					
3:30 PM	Mosquito Identification (Units 3&4, P)		*Coffee Break*	*Coffee Break*	*Coffee Break*	
4:00 PM		*Coffee Break*	Calculation of Malaria Transmission Parameters (Unit 8, P)	Data Analysis & Handling (Unit 8, P)	Data Handling (Unit 8, P)	*Coffee Break*
4:30 PM		Prepare for Night Biting & Light Trap Collection (Unit 6, P)			Prepare for Night Biting & Light Trap Collection (Unit 6, P)	Calculation of Malaria Transmission Parameters (Unit 8, P)
5:00 PM			Preparations for PSC (next morning) (Unit 6, P)			
5:30 PM		*Dinner*			Dinner	
Skip to 7:00 PM		Night Biting & Light Trap Collection (Unit 6, P)			Night Biting & Light Trap Collection (Unit 6, P)	
10:00 PM						

T: theoretical class; T-P: theoretical-practical class; P: practical

Table A-2. Course schedule (week 3)

Time	Day 1	Day 2	Day 3	Day 4
9:00 AM	Practical on WHO bioassays (Unit 10, P)	demonstration of vector control of tools/methods	Review of Theory	Students evaluation (test)
9:30 AM				
10:00 AM				
10:30 AM	*Coffee Break*	*Coffee Break*	*Coffee Break*	*Coffee Break*
11:00 AM	Practical on WHO bioassays (Unit 10, P)	demonstration of vector control tools/methods	Review of Theory	Course evaluation
11:30 AM				
12:00 PM				
12:30 PM	*Lunch*	*Lunch*	*Lunch*	*Lunch*
1:00 PM				
1:30 PM	Practical on CDC bottle (Unit 10, P)	demonstration of vector control tools/methods	Discussion on Practicals	Course Assessment
2:00 PM				
2:30 PM				Closing Ceremony
3:00 PM				
3:30 PM				
4:00 PM	*Coffee Break*	*Coffee Break*	*Coffee Break*	
4:30 PM	Practical on cone bioassays (Unit 10, P)	demonstration of vector control tools/methods	Discussion on Practicals	
5:00 PM				

T: theoretical class; T-P: theoretical-practical class; P: practical

Annex II Examples of Data Collection Forms for Larval and Adult Mosquito Surveys

A. LARVAL SURVEY DATA COLLECTION FORM

A.1. Identification of collection site

Region/district..... **Locality** .....

Geographic coordinates:

- **Latitude**.....
- **Longitude**.....

A.2. Characterization of the breeding site

Type

- **Permanent** ☐ **Semi-permanent** ☐ **Temporary** ☐

Origin of the water (*e.g.* rain, river, lagoon, man-made).....

Nature of the water collection (*e.g.* puddle, rice field, ditch).....

Characteristics of the water (*e.g.* clear, turbid, polluted, dark).....

- **Temperature**..... **pH**.....

Exposure to sunlight

- **Shaded** ☐ **Partially shaded** ☐ **Sunlit** ☐

Presence of vegetation (emergent, submerse, floating).

- **Emergent** ☐ **Submerse** ☐ **Floating** ☐

A.3. Sampling description

Sampling time (min)..... **Number of dips**.....

Presence of larvae

- **Anopheline** ☐ **Culicine** ☐ **Negative** ☐

A.4. Notes

.....

.....

Date..... **Hour of the collection**.....

Name of the collector.....

B. ADULT SURVEY DATA COLLECTION FORM

B.1. Identification of collection site

Region/District..... **Locality** .....

Geographic coordinates:

- **Latitude**.....
- **Longitude**.....

B.2. Type of collection

Human landing catches: **Indoor** ☐ **Outdoor** ☐

Resting collections: **Indoor** ☐ **Outdoor** ☐

Pyrethrum Spray Sheet collection ☐ **Exit trap** ☐

Other.....

B.3. characteristics of the collection site

Indoor collection

- **Type of house and construction materials**.....
- **Number of bedrooms**..... **Number of divisions**.....
- **Number of people that slept in the house on the previous night:**
  - **With bed net**..... **Without bed net**.....
- **Type of bed nets**
  - **Non-impregnated** ☐ **Impregnated** ☐ **LLIN** ☐
- **Date of the last time the house was treated by insecticide spraying**.....

Type and characteristics of the outdoor collection (e.g. animal shelter, vegetation).....
.....

B.4. Sampling description

Hour of the collection..... **Time duration**..... **Nº of collectors**.....

Presence of adult mosquitoes

- **Anopheline** ☐ **Culicine** ☐ **Negative** ☐

A.4. Notes

.....

.....

Name of the collector..... **Date**.....